Comparative molecular, physiological and proteomic analyses of maize and sorghum subjected to water deficit stress

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A thesis submitted in fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape

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Keywords

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Drought responses in sorghum and maize

Abstract

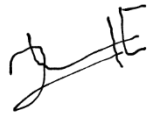
Drought is a major abiotic stress which causes not only differences between the mean yield and the potential yield but also yield variation from year to year. Although selection for genotypes with improved productivity under drought environments has been a central goal of numerous plant breeding programs, the molecular basis for plant tolerance towards drought stress is still poorly understood. Exposure of plants to this abiotic stress is known to trigger excessive formation of reactive oxygen species (ROS), which induce cell death and reduce growth. Part of the mechanism of plant responses to drought involves alterations in the expression of antioxidant enzymes and biosynthesis of different compatible solutes such as proline. Sorghum is regarded as generally more drought tolerant than maize, and it is a potential key model system for investigating the physiological and molecular mechanisms conferring drought tolerance. Comparative studies in crop plants to decipher differences in drought tolerance are essential for crop improvement to sustain a higher level of production, which in turn will improve food security, under severe drought conditions resulting from climate change. On this basis, the aim of this study is to determine molecular differences between *Zea mays* and *Sorghum bicolor* in response to drought stress in an attempt to identify novel biomarkers for drought tolerance. The physiological and molecular responses of maize and sorghum were studied for changes in growth, chlorophyll content, relative water content, ROS content, lipid peroxidation level, proline content, and antioxidant enzyme activity. Spectral Count Label-free Quantitation analysis was conducted to reveal the changes in protein profiles under drought in attempt to identify drought-responsive molecular mechanisms in the leaves of the two plant species. In this study, water deficit triggered mechanisms that resulted in overproduction of ROS in both *Zea mays* and *Sorghum bicolor*. However, *Sorghum bicolor* showed less oxidative damage under water stress compared to *Zea mays*. Drought-induced proline accumulation in the roots of *Sorghum bicolor* was associated with enhanced water retention. Significant changes were identified in the antioxidant enzyme activity between the two plant species in response to drought conditions. Proteomics results showed differing patterns for drought-responsive proteins in the two species. Together with the physiological, biochemical and proteomic profiling results between *Zea mays* and *Sorghum bicolor*, potential proteins and/or metabolic pathways underlying drought tolerance were identified. The findings obtained through this study provide insight towards understanding the molecular basis of crop drought tolerance.

DECLARATION

I declare that ”Comparative molecular, physiological and proteomic analyses of maize and sorghum subjected to water deficit stress” is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Ali Elnaeim Elbasheir Ali

Signature



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List of Abbreviations

1-D: One-dimensional gel electrophoresis

2-D: Two-dimensional gel electrophoresis

ABA: Abscisic acid

ACN: Acetonitrile

ADP: Adenosine diphosphate

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

APX: Ascorbate peroxidase

ASA: Ascorbic acid

ASH: Ascorbate

ATP: Adenosine triphosphate

B2: Riboflavin

BCA: Bicininchoc Acid

BSA: Bovine serum albumin

CA: trans-cinnamic acid

CAT: Catalase

CBB: Coomassie Brilliant Blue

CCAFS: Climate Change, Agriculture and Food Security

CGIAR: Consortium of International Agricultural Research Centres

CHAPS: 3-[(3cholamidopropyl) dimethylammonio]- propanesulfonate

DHA: Dehydroascorbate

DMRT: Duncan's Multiple Range Test

DMSO: Dimethyl sulfoxide

DOPA: Dihydroxyphenylalanine

DTNB: Nitrobenzoic acid

DTT: Dithiothreitol Cleland's reagent

EDTA: Ethylenediaminetetraacetic acid

FA: Formic acid

FDR: False discovery rate

GAA; Glacial acetic acid

GO: Gene ontology

GPX: Glutathione peroxidase

GR: glutathione reductase

GSH: Glutathione

GSSG: Glutathione disulfide

HILIC: Hydrophilic interaction liquid chromatography

HPLC: High-performance liquid chromatography

IEF: Isoelectric focusing

KCN: Potassium cyanide

LC-MS/MS: liquid chromatography-mass spectrometry and tandem mass spectrometry

MDA: Malondialdehyde

MDHA: Monodehydroascorbate

MMTS: Methylmethanethiosulphonate

MTT: 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NAD: Nicotinamide adenine dinucleotide

NADP: Nicotinamide adenine dinucleotide phosphate

NADPH: Nicotinamide adenine dinucleotide phosphate

NBT: Nitrotetrazolium Blue chloride

PA: Phosphatidic acid

PAGE: Polyacrylamide gel electrophoresis

PEP: Phosphoenolpyruvate

PLD: Phospholipase D

PMS: Phenazine methosulfate

PMSF: Phenylmethylsulfonyl fluoride

PPDK: pyruvate, phosphate dikinase

PS I: Photosystem I

PS II: Photosystem II

PSM: Peptide-spectrum matches

PTAL: phenylalanine/tyrosine ammonia-lyase

PVPP: Polyvinylpolypyrrolidone

Q-TOF: Quadrupole-time-of-flight

ROS: Reactive oxygen species

RPM: Revolutions per minute

RWC: Relative water content

SDS: Sodium dodecyl sulfate

SOD: Superoxide dismutase

TBA: Thiobarbituric Acid

TCA: Trichloroacetic acid

TCEP: tris (2-carboxyethyl) phosphine

TEAB: Triethylammonium bicarbonate

TEMED: N, N, N', N'-Tetramethylethylenediaminese

TFA: Trifluoroacetic acid

WD: Water-deprived

WW: Well-watered

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Chapter One

Literature Review

1.1 Introduction

Maize and sorghum are related crops which are members of the *Panicoideae* subfamily in the family of *Gramineae* (Schober and Bean, 2008).

Worldwide, maize (*Zea mays L.*) is the third major crop based on the harvested area (Ramirez-Cabral *et al*., 2017) and is the main food grain in Sub-Saharan Africa (Richard *et al.*, 2015). In South Africa, maize is considered as the most significant cereal crop and is produced across the country in varying environments. On average, approximately 8.0 million tons of maize is harvested in South Africa annually from roughly 3.1 million hectares of land. Almost one half of the production consists of white maize intended for human consumption (du Plessis, 2003). In developing countries, maize is consumed directly and considered as staple for at least 200 million people. Nevertheless, it is also processed into biofuel (bioethanol) and starch. Starch, for its part, is also converted to products such as sorbitol, dextrin, sorbic acid and lactic acid, and is found in domestic products such as beer, ice cream, syrup, glue, fireworks, ink, cosmetics, print and aspirin (du Plessis, 2003).

Sorghum is one of the most drought adaptive crops and the availability of its full genome sequence makes it a key model system for the study of physiological and molecular mechanisms underpinning drought tolerance (Mullet *et al*., 2002; Sabadin *et al.*, 2012; Sanchez *et al.*, 2002).

Sorghum is the fifth major grain in the world after maize, rice, wheat and barley, cultivated for various uses such as food, feed and biofuel (Ng'uni *et al.*, 2016). In Africa, it is the second most important cereal crop, given that approximately 20 million tons a year are produced on the continent, which is approximately one-third of the world's production (FAO, 2003).

Sorghum originated in the Northeast of Africa (Grenier *et al.*, 2004; Winchell *et al.*, 2017) and is unique in its adaptation to Africa's climatic conditions (Tonitto and Ricker-Gilbert, 2016), being able to tolerate long periods of limited water supply. Sorghum is processed in

Africa with a wide range of appealing and nourishing traditional foods such as semi-fermented bread, dumplings, couscous, fermented and unfermented porridges. In the highly competitive multinational business of beer production, sorghum has proven to be the best alternative to barley for the production of beer (Vunyingah and Kaya, 2016).

Global climate change has a major impact on the environment and socioeconomic development. The fundamental elements of agriculture (soil moisture, heat and sunlight) are influenced by climate change as it leads to variations in temperature, rainfall, and the occurrence of extreme climatic events such as drought (Xu *et al.*, 2017). With the limited adaptation and knowledge about molecular basis of drought tolerance, reduced agricultural productivity threatens food production and global food security (Heinemann *et al.*, 2017).

Drought is one of the most serious natural hazards in the world and its frequency and severity might be intensified in coming years due to global warming (Ortega-Gómez, Pérez-Martín and Estrela, 2018). Drought is the most significant factor limiting plant production in the world's agricultural fields (Sabadin *et al.*, 2012).

Improving and sustaining crop yield stability under water constrained conditions is important to ensure food security for the growing world population (Basu *et al.*, 2016). In water-vulnerable regions such as South Africa, screening of drought adaptive responses is essential to improving crop production under water deficit. However, through the evaluation of the physiological and molecular responses of crop plants under water-limited conditions, drought tolerance indexes could be obtained to provide insight towards crop improvement. Therefore, the **aim** of this work are to determine molecular differences between maize and sorghum in response to drought stress in an attempt to identify novel biomarkers for drought tolerance. **Objectives** include the assessment of morphological, physiological and molecular responses, as follows:

- Evaluating physiological responses of both cereal crops by determining the shoot fresh weights, shoot length, root length, relative water content (RWC), chlorophyll content and cell viability.
- Assessing molecular responses of the two cereal crops by measuring their antioxidant enzyme (SOD, APX, CAT and GR) activities, lipid peroxidation (MDA) and the level

of ROS (H_2O_2) and O_2^-.

- Reveal the changes in protein expression of the two cereal crops under drought using Differential Label-free Quantitative Proteomic Analysis.

1.2 Impact of drought on agriculture and the economy

Worldwide, 80% of the surface area of agricultural land relies on rainfall for water supply. The Consortium of International Agricultural Research Centres (CGIAR); through its Research Program on Climate Change, Agriculture and Food Security (CCAFS); has shown negative impact of climate change on crop production from the 2030s to several decades afterwards (Figure 1.1). According to current research and future drought forecasts, the yield of crops will be significantly reduced, which is a major threat to food security (Zargar *et al.*, 2017). Drought negatively impacts agricultural production as it prevents the crop from achieving its maximum potential yield (Mitra, 2001).

In South Africa, drought is one of the most serious phenomena severely affecting the country's agricultural economy. The effects of drought (first-order effects such as food security and secondary effects, such as increasing food imports from abroad) have a wide range of impacts on society (Benson and Clay, 1998). For example, maize is very sensitive to rainfall fluctuations because extended dry periods may lead to reduced grain formation and yield decline (Clay *et al*., 2003). The price of basic commodities increases during droughts as supplies are reduced.

Numerous South African households suffer from continual food insecurity and malnutrition. Approximately 14.3 million South Africans are exposed to food insecurity at any time (Food Pricing Monitoring Committee, 2003). South Africa's currency (Rand value) depreciation affects the price of maize, which gets worse when there is a grain scarcity during drought, for example in 2001-2002. This exacerbates the poverty level in susceptible communities.

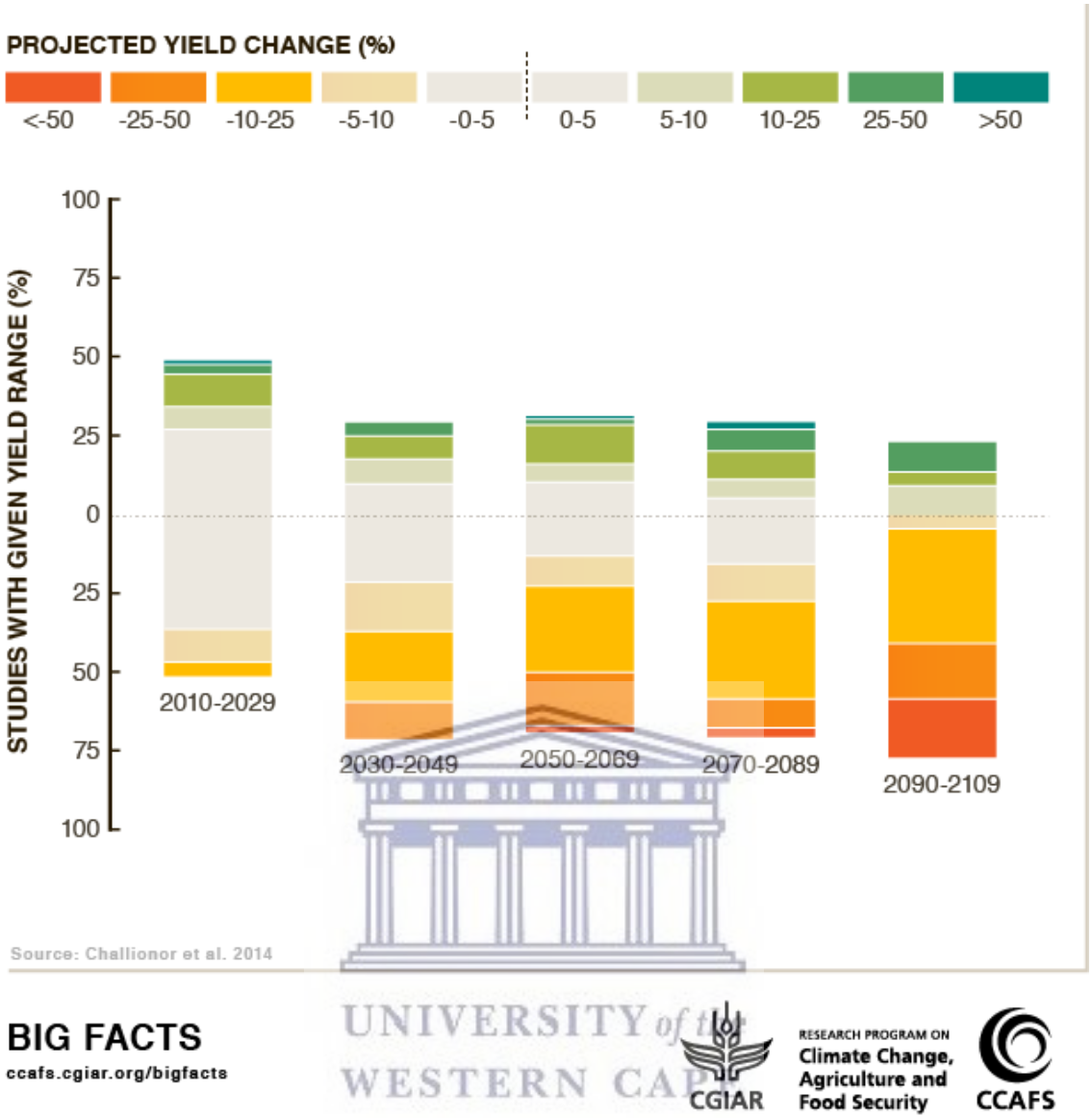


Figure 1.1 Climate change impacts on agricultural production and food security. Climate change will have a negative impact on crop yield from the 2030s onwards. Predictions for the period after the 2050s show a yield decline greater than 10%. Figure obtained from the Consortium of International Agricultural Research Centers (CGIAR)' Research Program on Climate Change, Agriculture and Food Security (CCAFS). https://ccafs.cgiar.org/bigfacts/#theme=climate-impacts-production

1.3 Plant responses to drought

Figure1.2 provides a summary of the effects of different drought levels and responses initiated

at these levels. Mild to moderate levels of drought influence the characteristics of stomata, resulting in biomass reduction. However, when drought becomes severe, it may affect photochemical efficiency and Rubisco activity, which affect physiological and biochemical processes essential for growth and survival (Xu, Zhou and Shimizu, 2010). Stomatal conductance enhances the leaf gas exchange (Gutschick, 2007) and controls the rate of leaf transpiration (Mcadam and Brodribb, 2014). On the other hand, changes in transpiration ratio can cause changes in plant water status (Xu, Zhou and Change, 2008).

Under drought conditions, photosynthesis and growth tend to be affected. This is because decreased stomatal conductance in attempt to maintain an adequate water status results in decreased CO_2 intake (Chaves *et al*., 2009). It has been shown plants exposed to a very severe level of drought eventually generate photoinhibitors and experience almost permanent stomatal closure (Flexas and Medrano, 2002). When drought is terminal, water availability in the soil is gradually reduced to a point where it eventually leads to premature plant death. In the case of intermittent drought, limited periods of insufficient water availability arising in several intervals during the growing season impact growth but are not severe enough to cause premature plant death although they impact yield (Neumann, 2008).

Understanding the interaction between the different plant responses at the physiological, biochemical and molecular level to drought stress is crucial to identify features that could improve crop tolerance to drought through the use of conventional breeding and transgenic strategies. In this sense, this review discusses the current understanding of plant responses to drought stress.

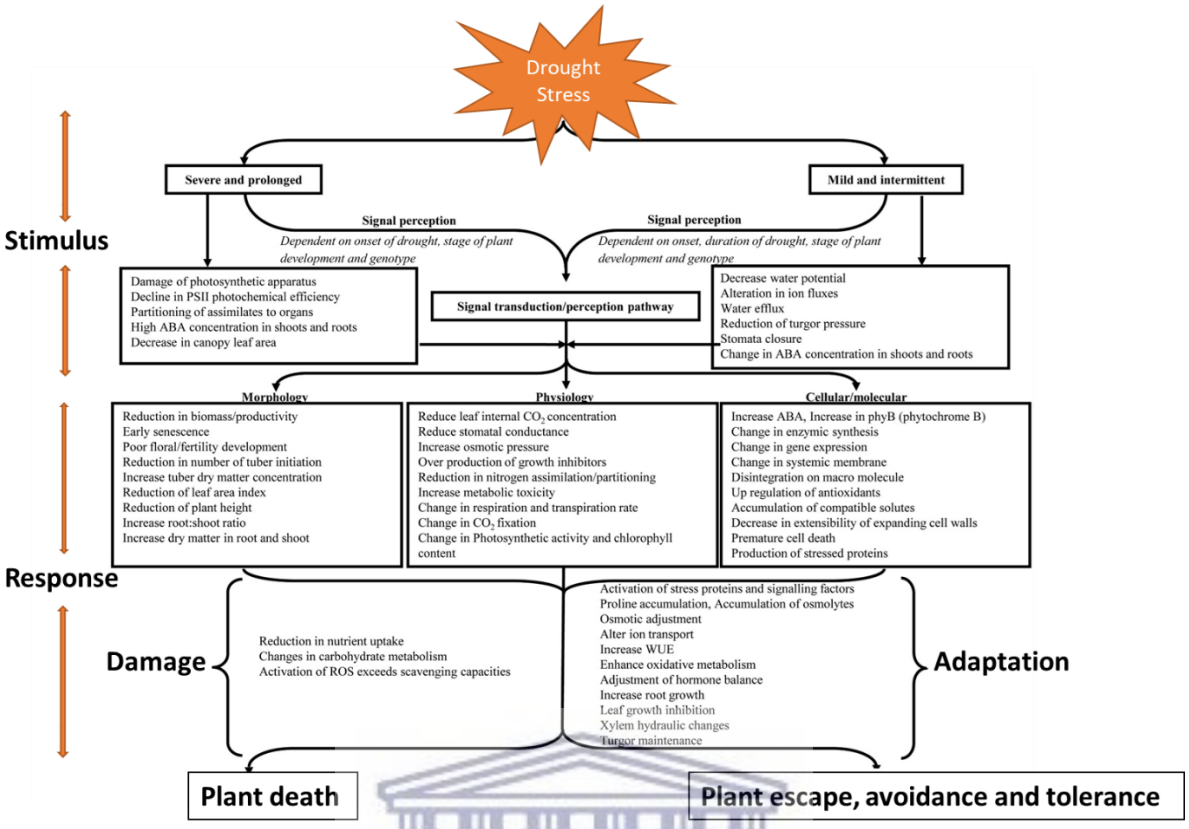


Figure 1.2 Different levels of drought influence the morpho-physiological and molecular responses, adapted from Obidiegwu (2015).

1.3.1 Physiological and morphological responses

Drought stress is a major factor reducing the growth and development of plants, which leads to inadequate flower production and grain filling, hence grain yield reduction. Water deficit significantly reduces cell expansion and cell growth, but osmotic regulation may maintain the cellular turgor pressure to assist the growth of plants under severe drought environments (Jaleel *et al*., 2009). A common adverse influence of water deficit on crops is the decline in fresh and dry weights (Anjum *et al*., 2011). Therefore, maintenance of biomass production under water restricted conditions is a desirable trait.

Lack of adequate water, which reduces soil water potential, reduces the size of individual leaves and the number of leaves per plant (Sinclair *et al*., 1986). The expansion of the leaf

surface depends on turgor pressure, temperature and assimilation of the nutrients (Shao *et al.*, 2008). The sharp reduction of the leaf surface is attributed to the suppressed expansion of the leaves, which also impacts on net photosynthesis (Lei *et al*., 2006).

1.3.2 Molecular responses

1.3.2.1 ROS biochemistry

Several reactive oxygen species (ROS) are produced in plants as by-products of aerobic metabolism (Apel and Hirt, 2004). ROS include free radicals such as superoxide anion (O_2^-), hydroxyl anion (OH^-), as well as non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These reactive oxygen species are known to play a dual role as both toxic and beneficial species depending on the concentrations of these species in plants (Mittler, 2017). When the concentration of these species is finely controlled, they acts as secondary messengers in intracellular signalling cascades mediating a number of processes in plant cells (Schieber and Chandel, 2014). Over-accumulation of these reactive oxygen species as a result of various environmental stresses, such as drought, can lead to severe cellular damage as a result of damage to proteins, lipids and DNA, leading to physiological dysfunction and cell death (Singh Gill *et al*., 2011) .

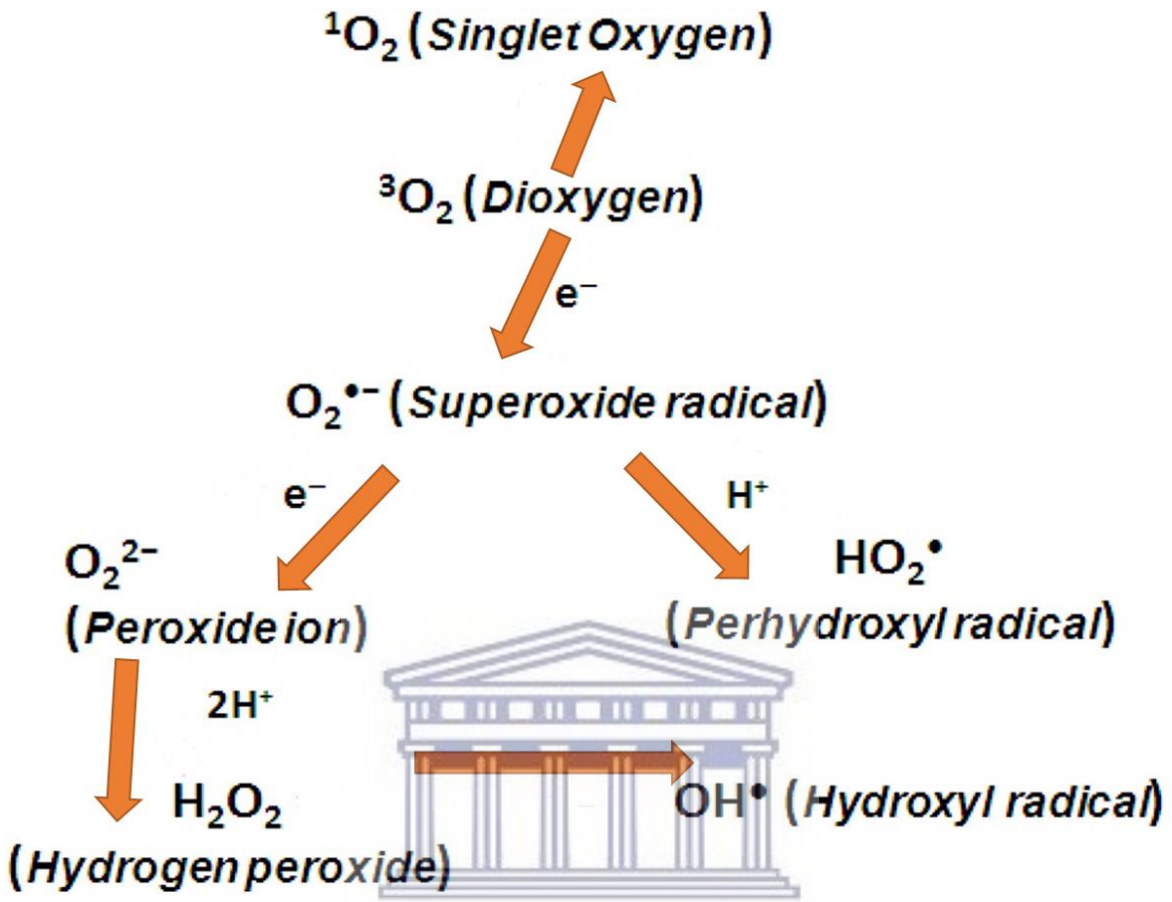


Figure 1.3 generation of reactive oxygen species through energy transfer adapted from Das and Roychoudhury (2014)

Hydrogen peroxide is one of the most stable ROS and is an essential signalling molecule regulating several metabolic functions (Sies, 2014). Hydrogen peroxide (H_2O_2) is produced from superoxide due to the catalytic activity of NADPH oxidases (Brand, 2010). H_2O_2 diffuses through cell membranes and tissues, initiating direct cellular processes such as cell shape changes, proliferation initiation and recruiting of immune responses (Slesak *et al*., 2007).

Superoxide ($O_2^{\bullet-}$) is continuously produced during photosynthesis in the reaction centres of photosystem I (PSI) and photosystem II (PSII) by partial reduction of molecular oxygen

(Pospíšil, 2012). Superoxide radicals ($O_2^{\cdot -}$) are toxic substances, produced in plants as a result of oxidative stress under adverse environments. Toxicity is due to their interaction with hydrogen peroxide (H_2O_2) to produce high reactive hydroxyl radicals ($OH^{\cdot}$) in the reaction (Xu *et al.*, 2015).

Hydroxyl radicals are the primary cause of protein and lipid peroxidation as well as nucleic acids damage during oxidative stress (Liszkay *et al*., 2004). Hydroxyl radicals ($OH^{\bullet}$) are generated from reaction of $O_2^{\cdot -}$ with H_2O_2 by an iron-catalysed reaction (Xu *et al.*, 2015). Singlet oxygen ($^1O_2$) is mainly formed during photosynthesis in the PSII reaction centre, by photodynamic activation of ground-state oxygen which reacts with chlorophyll (Fischer *et al*., 2013). The life cycle of the $^1O_2$ in the cell is measured to be approximately 3 microseconds (µs) (Gill and Tuteja, 2010). It is well established that carotenoids can efficiently minimize singlet oxygen production through several quenching mechanisms that disperse excessive light energy as heat (Ruban *et al.*, 2012). However, these regulatory mechanisms are limited. If these limits are exceeded, singlet oxygen may trigger lipid peroxidation reactions, which may lead to stress-induced photo-oxidation (Triantaphylides *et al*., 2008).

1.3.2.2 Oxidative stress and plant defence mechanisms

Drought is known to trigger excess accumulation of reactive oxygen species resulting in oxidative stress that lead to cell damage. To minimize the oxidative damage, plants activate the expression of genes encoding redox enzymes and enhance the production of non-enzymatic antioxidants as a form of defence against oxidative stress (Figure 1.4).

Enzymatic ROS-scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), catalase (CAT), and glutathione peroxidase (GR). The non-enzymatic antioxidant defences include ascorbate (ASH), glutathione (GSH), tocopherol, carotenoids and phenolic compounds.

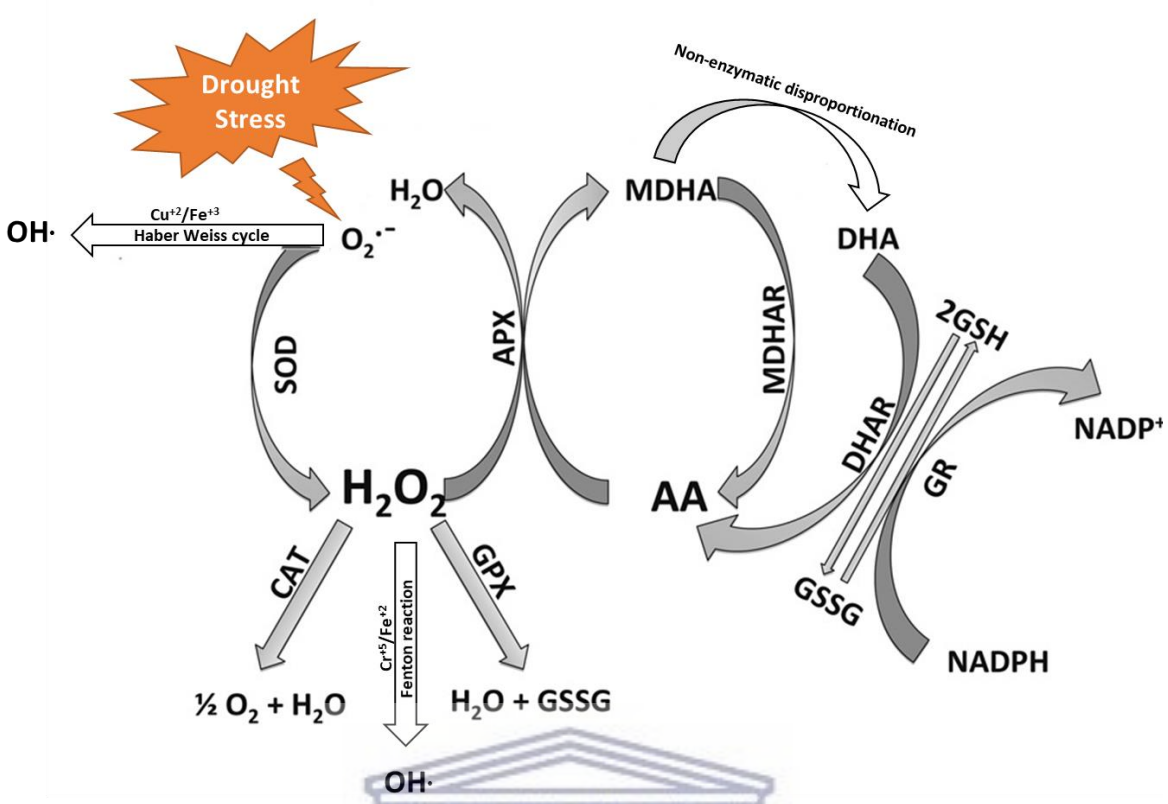


Figure 1.4 ROS and antioxidant defence mechanisms
Enzymatic ROS scavenging mechanisms include superoxide dismutase (SOD), catalase (CAT), the ascorbate-glutathione cycle, and the glutathione peroxidase (GPX) cycle. SOD converts superoxide to hydrogen peroxide. Hydrogen peroxide is detoxified into water by the CAT and ascorbate glutathione cycle. The former reaction requires an ascorbate and GSH regeneration system. Ascorbate peroxidase oxidises ascorbate into MDHA, which can be reduced to ascorbate by MDHA reductase using NADPH as a reducing agent with spontaneous production of Dehydroascorbate (DHA). DHA reductase reduces (DHA) ascorbate. This reaction is driven by the oxidation of GSH into GSSG. Finally the cycle is closed by Glutathione reductase (GR) regenerating GSH from GSSG with the help of NADPH as a reductant. The GPX cycle also detoxifies H_2O_2 to water using GSH directly as a reducing agent. The GPX cycle is closed by converting oxidised GSSG again into GSH by GR using NADPH as a reducing agent. This mode of the enzymatic ROS scavenging system results in improved plant tolerance against oxidative stress (Gill and Tuteja, 2010).

1.3.2.2.1 Enzymatic Reactive Oxygen Species-Scavenging Pathways

Superoxide dismutase (SOD) is considered to be the first line of defence in plants against ROS

(Alscher *et al.*, 2002). This enzyme converts (O_2^-) to H_2O_2 and was first found in maize, which contained six different isozymes of SOD (Scandalios, 1993; Turk and Erdal, 2015). SODs feature a metal ion in their active sites and it is on this basis that SOD can be classified into copper/zinc (Cu/ZnSOD), manganese (MnSOD) as well as iron (FeSOD) SOD isoforms (Kingston-smith and Foyer, 2000). Up-regulation of SOD is associated with oxidative stress triggered by abiotic stresses and plays an essential role in the protection of plants against oxidative damage (Hasanuzzaman *et al.*, 2014).

Ascorbate peroxidase (APX) as an antioxidant enzyme which plays a vital role in the metabolism of H_2O_2 in plants. This enzyme is a central component in the detoxification of H_2O_2 in water-water and ascorbate-glutathione cycles, using ascorbate (AsA) as its specific electron donor (Shigeoka *et al.*, 2002). The APX family comprises of five different APX isoforms including thylakoid (tAPX), peroxisome (pAPX), stromal (sAPX) as well as cytosolic (cAPX) forms (Goraya and Asthir, 2016).

Catalase (CAT) was the first antioxidant enzyme characterized. Plants contain multiple catalase isozymes which are mainly located in the peroxisomes (Mhamdi *et al.*, 2010). Catalase enzymes convert 2 H_2O_2 molecules into O_2 + 2H_2O (Kingston-Smith and Foyer, 2000; Anjum *et al.*, 2016). Catalases also have the ability to oxidize other substrates such as ethanol, methanol, formaldehyde, and formic acid (Komarova *et al.*, 2014; Dorokhov *et al.*, 2015).

Glutathione peroxidases (GPX) are a large family with multiple isozymes which play a key role in the detoxification of reactive oxygen species during oxidative damage (Roxas *et al.*, 2000). GPX utilizes GSH to reduce H_2O_2 and cytotoxic hydroperoxides into alcohols (Banerjee and Vats, 2014; Passaia and Margis-pinheiro, 2015). Glutathione reductase (GR) has been identified in several plant tissues and is mainly localized in the chloroplast stroma but is also found in the mitochondria, cytosol and peroxisomes (Singh *et al.*, 2013). GR has a fundamental role in plant protection against ROS-induced oxidative stress, using NADPH to reduce glutathione disulphide (GSSG) to the sulfhydryl form (GSH) and is therefore essential to maintain the reduced glutathione pool (Banerjee and Vats, 2014).

1.3.2.2.2 Non-enzymatic antioxidant defence systems

Glutathione (GSH) is considered to be the most important protection in plants against reactive oxygen species during oxidative stress (Das and Roychoudhury, 2014). Glutathione acts as a ROS-scavenging antioxidant in several ways. In the ascorbate-glutathione cycle, GSH is utilized to reduce dehydroascorbate (DHA). Moreover, GSH is also oxidized to glutathione disulphide (GSSG) during the reaction (Shao *et al.*, 2008). GSSG can also be converted back to GSH by glutathione reductase using NADPH as a reducing agent (Apel and Hirt, 2004).

Ascorbic acid (AsA) plays a fundamental role in the plant antioxidant defence system which protects against H_2O_2 and other toxic oxygen radicals (Foyer *et al*., 1994). As a reducing agent, AsA is involved in the ascorbate–glutathione cycle, where two molecules of AsA are utilized by APX to reduce H_2O_2 to water, with simultaneous production of monodehydroascorbate (MDHA) (Villegas *et al*., 2002), which is a short-lived radical and may be disproportionate to dehydroascorbate (DHA) and AsA (Kwon *et al*., 2003)

Tocopherols are antioxidants are found in all parts of plants (Dziki, 2014). They play a crucial role in plant protection mechanisms by detoxifying ROS (Ighodaro and Akinloye, 2018). Of the four isomers (α, β, γ, and δ), α-tocopherol is the most crucial isoform and is essential for the protection of the chloroplast membrane against photo-oxidative deterioration (Fryer, 1992). The antioxidant activity of α-tocopherols is based on their capability of quenching singlet oxygen by the mechanisms of charge transfer (Trozzolo *et al.*, 1974; Fukuzawa *et al.*, 1997, 1998).

Carotenoids (Car) are photosynthetic pigments found in plants and microorganisms, with over 6000 carotenoids occurring in nature (Lee and Schmidt, 2002). The fundamental antioxidant property of carotenoids is based on the structure of a conjugated double which enables these molecules to delocalize unpaired electrons (Young and Lowe, 2001) which are mainly responsible for β-carotene's ability to inhibit ($^1O_2^-$)-dependent lipid peroxidation (Mortensen and Skibsted, 1997).

1.3.2.2.3 Osmolyte accumulation as a defence mechanism during stress

The accumulation of compatible and inorganic solutes is considered to be a basic strategy that plants developed to protect themselves under abiotic stress conditions (Chen *et al*., 2007). Plants subjected to abiotic stress accumulate intercellular organic osmolytes such as proline, glycine betaine, valine, aspartic acid, betaine, glucose, fructose and sucrose (Burg and Ferraris, 2008). The accumulation of these osmolytes serves as an adaptive response in mediating osmotic adjustment that maintains the water status of the cell and protects subcellular structures (Hare *et al*., 1998).

1.3.3 Plant adaptations to drought stress

Under drought stress, plants exhibit various morphological, physiological and molecular adaptations (Bohnert, 1995). In agriculture, resistance to drought refers to the ability of a crop to produce close to its potential yield with minimal loss under water deficit, with adaption strategies that enable it to escape, avoid or tolerate water stress (Mitra, 2001).

'Drought escape' is the ability of a plant to complete its life cycle before serious water stress can affect plant growth and yield. Thus, plants do not experience severe water stress, as they are able to achieve rapid vegetative growth and seed reproduction before soil moisture depletion. These plant adaptations do not involve any special morphological, physiological, or biochemical traits (Yıldırım and Kaya, 2017). 'Drought avoidance' is the ability of plants to maintain favourable internal water content through various adaptive traits involving the minimization of leaf water loss by reducing transpiration during drought (Szota *et al.*, 2017). 'Drought tolerance' is the ability of plants to cope with low internal water content through adaptive features. These adaptations involve maintenance of cell relative water content through osmotic adjustment, increased anti-oxidative capacity and enhancing protoplasmic tolerance (Yoshimura *et al.*, 2008).

1.4 Advances in crop proteomics and sustainable agriculture for tomorrow

The molecular responses of plant to drought stress is complex therefore, it would be remiss to studying gene expression or biochemical pathways in isolation. This is due to the fact that many biological processes occur in the cell including enzymatic processing, splicing events and/or post-translational changes which alter the expression of genes and protein turn-over (Tan *et al*., 2017). More so, the level of a particular mRNA does not always correlate with the protein abundance. The mRNA of highly transcribed genes can be quickly degraded or translated inefficiently, leading to disproportionate amounts of mRNA and protein. Furthermore, only a part of a particular mRNA pool is recruited to the ribosome assembly for translation (Kim *et al*., 2014). In this sense, high-throughput proteomics techniques are becoming increasingly important as a powerful omics-based tool which accurately detects changes in the protein expression (Zhang *et al*., 2007; Kim *et al.*, 2014).

The term "proteomics" refers to the universal study of gene expression at the protein level. Advances in proteomic techniques have enabled reliable analysis of biological mixtures, which led to identification of a wide spectrum of proteins in living organisms (Kim *et al*., 2014). This feature is particularly useful for crop science as it may offer information not only on nutritional significance, but also on yield level and the influence on these factors by unfavourable conditions such as drought stress (Chen and Harmon, 2006). Proteomics, one of the most significant post-genomic era tools (Chevalier, 2010) may provide a comprehensive identification of drought-responsive proteins in plants (Wang *et al.*, 2016).

The understanding of the cellular proteome makes it easy to identify changes in the expression of proteins during treatment and different growth conditions (Salekdeh and Komatsu, 2007). Traditional methods such as high-resolution two-dimensional polyacrylamide gel electrophoresis (2-DE) have been used to analyse the potential alterations in protein expression (Issaq and Veenstra, 2008 ; Magdeldin *et al*., 2012 ; Gong and Wang, 2013) subsequent to changes in environmental conditions. However, this method becomes limited as it has a low dynamic range, is ineffective when analysing insoluble proteins or proteins that have very low or high molecular weight and it is also limited in terms of reproducibility (Panchaud *et al.*, 2008).

In recent years, an alternative non-gel-based protein analysis technique referred to as Label-free Peptide Quantification has been developed for comparative proteome analysis (Katz *et al*., 2010). These improvements in technologies have made proteomics a very active area of research for biomarker identification and validation (Wang *et al*., 2008). Label-free proteomics is a protein quantification tool which employs liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection (Ono *et al*., 2006). It is used routinely for comparative proteomic analyses (Zhou *et al*., 2012). Therefore comparative analysis of differentially expressed proteins under drought-induced conditions provides benefits for understanding the molecular responses of plants to drought (Kim *et al.*, 2014).

Despite the fact that various studies show a link between ROS, antioxidant activity and drought responses in maize and sorghum moreover, despite the already reported studies on proteomic analyses of the responses of these two species to drought, no comparative studies have been done to understand the molecular basis for the differences in drought responses between maize and sorghum. This is a major gap in the understanding of drought responses at the molecular level across these two species. Filling this knowledge gap is important since it would establish clearer understanding of the determinants of the better drought tolerance in sorghum than maize. This has potential to improve drought tolerance in both species.

Chapter Two

Materials and Methods

2.1 Plant growth Conditions

Sorghum [*Sorghum bicolor* (L.) Moench cv. Superdan] and maize [*Zea mays* (L.) cv. Border King) seeds were surface sterilized in 0.35% (v/v) sodium hypochlorite for 10 minutes and then washed five times with distilled water. Seeds were then imbibed water for 16 hours in aerated 10 mM calcium sulphate ($CaSO_4$; Sigma). After rinsing with distilled water, the seeds were then incubated in the dark (plastic container covered with foil at room temperature in a moist growth medium with nutrient solution) for 3 days to germinate. Seedlings were sown in 10 cm diameter pots with a height of 100 cm, filled with 18 L of Promix Organic (Windell Hydroponics, South Africa) wetted to saturation with water containing 1% fertilizer] (v/v) [Nitrosol®, Envirogreen (Pty) Ltd)] and grown under greenhouse conditions (23˚C under 16/8 h light/dark cycle and photosynthetic photon flux density of 400 $\mu mol.m^{-2}.s^{-1}$ during the light phase). Plants were irrigated every second day with 500 ml of water until the V1 stage of development (one leaf with a visible collar).

2.1.1 Plants treatment

Plants at the V1 stage were selected for all experiments. The well-watered plants (control) of both sorghum and maize were provided with 500 ml of water every two day until the day of harvest (V8 stage of growth) and 100 ml (20%) of that amount of water was applied in water-deprived plants once a week until the V3 stage of development. At this stage, a complete water deprivation regime was then applied for the water-deficit plants (i.e. no more water supply) until the signs of drought stress were observed (two to four old leaves turned brown) then the four youngest leaves of plants were harvested. For maize, this occurred 40 days from the day when no more water was applied; sorghum required 55 days. The harvested leaves were immediately frozen in liquid nitrogen and finely ground into a powder using a cold pestle and mortar in liquid nitrogen, except for leaf sections that were used for measurement of fresh

weight, cell viability and $O^{\cdot}_2$. Powdered plant material was then stored at -80 ˚C for further use.

2.2 Relative water content

The relative water content was measured in triplicate using the youngest fully expanded leaves of control and water-deprived plants. A 10 cm cutting from the tip of each leaf was made using a sterile surgical blade. The fresh weights of the plant cuttings were measured. The turgid weight was determined by weighing the leaves after 4 hours of incubation in Petri dishes containing distilled water under ambient light. The leaves were then dried in a drying oven for 48 hours at 60 °C, then immediately transferred into a desiccator and their dry weights were determined.

2.3 Chlorophyll Assay

A method described by Tait and Hik (2003) was used to estimate chlorophyll content. Leaf tissue (100 mg) from each species was mixed with 5 ml of 99.5% dimethyl sulfoxide (DMSO; Sigma). Three replicates of each species were incubated at 35 ˚C for three hours in the dark. 200 µl aliquots of chlorophyll extract was transferred to a 96 plate and absorbance readings were recorded at a wavelength of 649 and 665 nm. DMSO was used as a blank. Chlorophyll concentrations were then calculated using (Wellburn, 1994) equations, $Chl_a = 12.19A_{665} – 3.45A_{649}$ and $Chl_b = 21.99A_{649}$ $5.32A_{665}$.

2.4 Trichloroacetic Acid (TCA) Extraction

Trichloroacetic acid (TCA) extraction was used to obtain the non-protein extract that is used to estimate the concentration of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). A 5x volume of 6% trichloroacetic acid (TCA; Sigma) was added to 100mg of frozen ground plant material. The solution was then centrifuged at 13000 x g for 15 minutes at 4˚C after a brief vortex. The supernatant was transferred to a new sterile Eppendorf tube and used for determining the MDA and H_2O_2 content.

2.5 Determination of Lipid peroxidation degree

Malondialdehyde (MDA) is a natural by-product of lipid peroxidation, and its quantitative assessment is generally used as a measure of lipid peroxidation. A method described by Dhindsa *et al*. (1981) was used to measure the cell membrane lipid peroxidation of maize and sorghum. An aliquot (200 µl) of TCA extract was added to 400 µl of a solution containing 20% TCA and 0.5% thiobarbituric acid (TBA; Sigma). The mixture was briefly vortexed and boiled at 90 ˚C for 20 minutes. After 10 minutes of incubation on ice, the solution was centrifuged at 12 000 x *g* for 5 minutes. After centrifugation, 200 µl of the solution was transferred into a microtiter plate. The absorbance was then measured at 532 nm and 600 nm wavelengths. The data obtained were used to measure the lipid peroxidation level.

2.6 Evaluation of Cell Viability

A modified method described by Sanevas *et al*. (2007) was used for cell viability measurements. The second youngest leaf from three different plants of each treatment (well-watered and water-deprived) was used for the assay. A 1 cm^2 leaf cutting was placed in a 1.5 ml tube covered with foil, and stained with 0.25% Evans Blue (Sigma) at room temperature for 30 minutes. Following staining the leaf material was rinsed with distilled water to remove Evans Blue, which did not penetrate the plant cells. Leaf materials were then transferred to a new tubes filled with distilled water. The materials were incubated overnight at room temperature in order to thoroughly eliminate any free dye. After removal of the water, samples were then incubated in 1.5 ml of 1% sodium dodecyl sulfate (SDS; Sigma) at 65˚C for 1 hour to extract Evans Blue. After centrifuging the samples at 13000 x *g* for 5 minutes, three 200 µl aliquots of the supernatant were transferred to microtiter plate. Absorbance readings were measured at 600 nm.

2.7 Reactive oxygen species (ROS) Measurements

2.7.1 Hydrogen peroxide

A standard curve was prepared with known hydrogen peroxide (H_2O_2; Sigma) concentrations to quantify hydrogen peroxide in both leaves and roots of maize and sorghum. Ten µM H_2O_2

was mixed with varying amounts of dH_2O, 0.5 M potassium iodide (KI; Sigma) and 20 mM potassium phosphate (K_2HPO_4; Sigma), pH 5, to a total volume of 200 µl. Three replicates of both samples and standards were prepared. An experimental mixture was made by mixing 50 µl of TCA extract with 100 µl KI and 50 µl of K_2HPO_4 to give a total volume of 200 µl. Absorbance was then measured at 390 nm and the extinction coefficient 39.4 mM^{-1} cm^{-1} was used to determine the content of hydrogen peroxide.

2.7.2 Superoxide

The superoxide content was determined using a modified method of Bates *et al*. (1973). A cm^3 squares of fresh leaf materials and 4 cm cuttings of root (from the tip) were made from the two plant species under the different water conditions and placed into an Eppendorf tube containing 10 mM potassium cyanide (KCN; Sigma) (for inhibiting Cu / Zn SODs), 10 mM H_2O_2 (inhibiting Mn and Cu/Zn SODs), 2% SDS (inhibiting Mn and Fe SODs) and 80 µM Nitrotetrazolium Blue chloride (NBT; Sigma). The tubes were then filled to a volume of 800 µl using 50 mM potassium phosphate (KPO_4; Sigma) and incubated for 20 minutes at room temperature. After the incubation was completed, the plant material was crushed with a small pestle, then the tubes were centrifuged at 13000 x *g* for 10 minutes. The supernatant obtained was then transferred to a clean 1.5 ml tube, and 200 µl was loaded onto a 96-well plate. Absorbance was read at a wavelength of 600 nm. An extinction coefficient of 12.8 mM^{-1} cm^{-1} for NBT was used to measure super oxide content.

2.8 Free proline Content

Free proline content was determined using a modified method of Bates *et al*. (1973). A 100 mg of plant material was homogenized with 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at 13000 x *g* for 10 minutes. 2 ml of the supernatant was added to a 1.5 ml microcentifuge tube containing 2ml of cooled acid-ninhydrin (prepared by dissolving 1.25 g ninhydrin [$C_9H_6O_4$; Sigma] in 30 ml of glacial acetic acid [GAA; Sigma] and 20 ml of 6 M phosphoric acid [H_3PO_4; Sigma] in a water bath at 50°C) and 2 ml of glacial acetic. The reaction solution in the tubes was incubated in a water bath for 1 hour at 100°C and cooled immediately in an ice bath. Proline was then extracted by mixing 4 ml of 99.9% toluene

($C_6H_5CH_3$; Sigma) with the reaction mixture. The chromophore containing toluene was then transferred into a clean tube and warmed at room temperature. 200 µl of the chromophore containing toluene was loaded on a microtiter plate, and absorbance was recorded at 520 nm. Toluene was used for a blank. The proline content was estimated from a standard curve performed by using purified proline and calculated on the basis of plant material weight as follows: [(µg proline / ml × ml toluene) / 115.5 µg / µ mol] / (g sample) / 5] = µmoles proline / weight in g.

2.9 Total Protein Extraction

Crude extracts were obtained by adding 200 mg of frozen ground plant tissue to a 1.5 ml microcentifuge tube. The extract was homogenized with 400 µl of protein extraction buffer [40 mM phosphate buffer at pH 7.4; 1 mM Ethylenediaminetetraacetic acid (EDTA; Sigma) and 5% (w/v) Polyvinylpolypyrrolidone (PVPP; Sigma)]. After vortexing, the homogenate was centrifuged at 13000 x *g* for 20 minutes at 4˚C to pellet the plant material, and the supernatant was gently transferred to a clean microcentifuge tube to be used for the antioxidant enzyme assays. The Bradford assay was then used to determine the concentration of the extracts.

2.10 Quantification of Protein

Protein concentration was estimated using the method of Bradford (1976). The protein standard curve was generated using 1mg/ml of bovine serum albumin (BSA), distilled water and Bradford reagent. A mixture of 1 µl protein sample, 9 µl dH_2O and 190 µl Bradford regent (Biorad) was mixed together and incubated for 10 minutes at room temperature. After incubation, the mixture was loaded to a 96 well-plate and the absorbance readings were recorded at 595 nm using a UV-visible spectrophotometer. The assay was done in triplicate. The concentration of the protein samples were calculated using the equation derived from the standard curve.

2.11 Native-PAGE for Antioxidant Enzymes Activity

The staining of all in-gel assays (APX, CAT, SOD and GR) was performed using non denaturing discontinuous gel electrophoresis formed of 15 % separating gel [except for CAT (in which case the separating gel was a 7.5 % gel)] and 5 % stacking gel. The resolving gel consisted of 40% acrylamide/bis-acrylamide (Sigma), 1.5 M Tris (Sigma, pH 8.8), 10% ammonium persulfate (APS; Sigma)), 4 µl N, N, N', N'-Tetramethylethylenediaminese (TEMED; Sigma) and distilled water made up to a total volume of 10 ml. The same concentrations were used to prepare the stacking gel except for Tris (1 M at pH 6.8) and TEMED 5 µl in total volume of 5 ml. The gels were allowed to solidify at room temperature for 15 minutes. After the gel preparation, 100 µg of protein samples were mixed with 25 µg (1x loading dye) except for APX (50 µg) and the mixture was then loaded into each well. The gels were electrophoresed with a running buffer containing 25 mM Tris, 192 mM glycine and [2 mM ascorbic acid (AsA; Sigma) only for APX] under cold conditions (4˚C) at 80 mV until the loading dye reached the bottom edge of the gel except for CAT (the gel was kept running for 2 hours after the dye run off the gel). Finally, the gels were stained for specific activity in triplicate.

2.11.1 Ascorbate peroxidase (APX)

2.11.1.1 In gel-Assay

Modification to the method previously described by Seckin *et al*. (2010) was followed in order to determine APX isoforms activity. Following electrophoresis, the gel was allowed to equilibrate in 50 mM potassium phosphate buffer (KPO_4) at pH 7 contains 2 mM ascorbic acid. The gel was then incubated once in 50 mM KPO_4 at pH 7.8 containing 4 mM ascorbate for 10 minutes and 2 mM H_2O_2 and 50 mM KPO_4 at pH 7.8 for 1 minute. All the incubation steps above were done in the dark on a shaker. Finally, the gel was stained in 50 mM KPO_4 pH 7.8 containing 0.5 mM NBT and 28 mM TEMED, exposed to light on a light box until the APX activity was observed as pale zones on a dark blue background.

2.11.1.2 Spectrophotometric Assay

A modified method of Nakano and Asada, (1981) was used to determine the ascorbate peroxidase total activity. The reaction solution was prepared by adding 50 µg protein sample, 2 mM ascorbate that was dissolved in 50 mM potassium phosphate (KPO_4) buffer pH 7, 0.1 mM EDTA and 10 mM H_2O_2 (was added last to start the reaction) made up to a final volume of 200 µl with de-ionised water. Once the H_2O_2 was added the absorbance readings were immediately measured at 290 nm for 1 min. APX total activity was then determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

2.11.2 Catalase (CAT)

2.11.2.1 In gel-Assay

CAT activity staining procedure was described by Yamashita *et al.*, (2007). Following electrophoretic separation, the gel was first thoroughly washed with distilled water for 30 minutes, and the water was discarded every 10 minutes. After thorough washing, the gel was incubated for 20 minutes with 0.006 % H_2O_2 on a shaker in the dark. The gel was finally stained with 3.3 % ferric chloride ($FeCl_3$; Sigma) and 3.3 % potassium ferricyanide ($K_3[Fe(CN)_6]$; Sigma) on a light box to visualize the isozyme bands.

2.11.2.2 Spectrophotometric Assay

The catalase total activity was assayed by measuring the consumption of hydrogen peroxide using the modified method described by Luck, (1965). A reaction mixture consisting of 50 µg protein extract, 1.5 mM EDTA and 50 mM potassium phosphate buffer (pH 7) was prepared. Immediately after initiating the reaction by adding 1 mM H_2O_2, the absorbance was read at 240 nm. The calculations were done based on the decrease in absorbance (ε = 39.4 mM^{-1} cm^{-1}), and CAT activity was in units where one unit of CAT enzyme converts 1 µmole of H_2O_2 per minute.

2.11.3 Superoxide dismutase (SOD)

2.11.3.1 In gel-Assay

SOD iso-enzyme activity was determined on native-PAGE, and the staining was performed according to the method first described by Beauchamp and Fridovich, (1971) with slight modifications. Three gels were electrophoresed for this assay; two gels were used to identify the two SOD isoforms (Mn-SOD and Cu-Zn SOD). The gels were transferred into 3 different containers for the staining. Prior to staining, the first gel was incubated in 50 mM potassium phosphate (pH 7.8) buffer while the other two gels were incubated in 50 mM potassium phosphate buffer, pH 7.8 containing either 3 mM KCN or 5 mM H_2O_2 for 15 minutes. The gels were allowed to incubate in a solution consisting of 50 mM potassium phosphate (pH 7.8) buffer and 2.5 M NBT for 10 minutes. The two incubation steps above were done in the dark with shaking. The second solution was discarded, and the gels were stained with 50 mM potassium phosphate (pH 7.8) buffer containing 28 mM riboflavin and 28 mM N, N, N, N-tetramethylethylenediamine (TEMED), on a light box until SOD achromatic bands were developed.

2.11.3.2 Spectrophotometric Assay

The modified method of Beauchamp & Fridovich (1971) was used to measure Superoxide dismutase (SOD) total activity. 50 µg of protein samples was mixed with 50 mM phosphate buffer KPO4 (pH 7.8), 0.1 mM EDTA, 2 µM riboflavin (B2), 13 mM L-methionine and 75 µM nitroblue tetrazolium (NBT); and the mixture was allowed to incubate 10 minutes on a light box at room temperature to initiate the reaction. A blue color was formed, and absorbance was read at 590 nm. One unit of superoxide dismutase activity represented the amount of enzyme causing 50 % inhibition of NBT.

2.11.4 Glutathione reductase (GR)

2.11.4.1 In gel-Assay

Evaluation of GR isoforms activity was determined according to a modified method as reported by Rao *et al*., (1996). The gel was equilibrated in 50 mM TRIS-HCl (7.9) buffer

containing 2 mM nitrobenzoic acid (DTNB; Sigma), 4 mM glutathione disulphide (GSSG; Sigma) and 1.5 mM nicotinamide adenine dinucleotide phosphate (NADPH; Sigma). The presence of GR isoforms were visualized by staining the gel at 30°C with 50 mM TRIS-HCl (pH 7.9) buffer containing 0.6 mM 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; sigma) and 0.8 mM phenazine methosulfate (PMS; Sigma).

2.11.4.2 Spectrophotometric Assay

A modified method of Foyer and Halliwell (1976) was used to estimate the total activity of glutathione reductase (GR). The activity was determined by preparing a reaction mixture of 0.2 M KPO4 buffer (pH7) containing 10 mM GSSG and 50 µg protein. Subsequently, 1 mM NADPH was added to initiate the reaction. A decrease in absorbance at 340 nm for 1 minute was recorded, and GR activity was expressed as units (µmol of NADPH oxidized per minute) per mg of protein.

2.12 (SDS/Phenol) Extraction and 1D SDS-PAGE

Total protein was extracted using a modified method described by Wang *et al*. (2006) for proteomic analyses. Protein extraction was done in five biological replicates from well-watered and water-deprived plants of each species (maize and sorghum). Plant tissue (1g) was pulverized into a fine powder with 0.5 g Polyvinylpolypyrrolidone (PVPP) to precipitate "unwanted" polyphenolic compounds, which will "disturb" further analysis (gels and/or LC/MS) using liquid nitrogen and placed into a Falcon tube. The mixture was then suspended in 2 ml 10 % TCA:acetone (w/v). After homogenization, the resulting homogenate was then transferred into two different 2 ml tubes (one for 1D analysis and one for label-free analysis) and centrifuged at 13,000 x *g* for 20 minutes at 4°C; the supernatant was discarded. The pellet was washed twice with 80 % (v/v) methanol (CH_3OH; Sigma) made in 0.1 M ammonium acetate (Sigma). The supernatant was discarded, and the pellet was washed with 80 % (v/v) acetone. After each washing step, the sample was centrifuged for 10 min at 13,000 x *g* and supernatant was decanted. The pellet was then allowed to air-dry under a vacuum and suspended in 0.5 ml dense sodium dodecyl sulphate (SDS) buffer [(10 % (w/v) SDS, 0.1 M Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 5 % (v/v) 2-

mercaptoethanol (BME; Sigma), 30 % (w/v) sucrose (Sigma)]. To the suspension, 0.5 ml of phenol (Tris-buffered, pH 8.0; Sigma St. Louis, MO, USA) was added. The mixture was mixed thoroughly and centrifuged at 13,000 x *g* for 20 min at 4˚C. The upper phenol phase was then transferred into a clean centrifuge tube. The tubes were filled with pre-cooled methanol [80 % (v/v), made in 0.1 M ammonium acetate] and incubated overnight at 4 ˚C to precipitate the protein. After precipitation, the proteins were then pelleted by centrifugation at 13,000 x *g* for 20 min at 4˚C, then washed once with cold methanolic ammonium acetate and cold 80 % (v/v) acetone. Finally, the pellet was dried in a vacuum. The pellet, which was used for 1D gels, was dissolved in 100 µl isoelectric focusing (IEF; Sigma) buffer containing 7 M urea, 2 M thiourea, 4 % (w/v) 3-[(3cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS; Sigma) and 20 mM dithiothreitol (DTT; Sigma) and sonicated for 30s in a water bath at 25°C. The other set of pellets were used for label-free analysis. The protein concentration was determined using Bradford assay as described in section (2.10). Following the extraction, the quality of the protein extracts were evaluated by separating them on 1D SDS-PAGE.

2.13 Label-free mass spectrometry-based protein quantification

This study aimed to conduct label-free quantitative shotgun proteomics for the evaluation of the differentially expressed proteins in maize and sorghum under drought stress. A total of 20 samples (5 biological replicates of each in maize and sorghum under both well-watered and water-deprived conditions) were used for proteomic analyses. Samples were digested using an automated hydrophilic interaction liquid chromatography (HILIC) magnetic bead-based workflow; afterwards, peptides were then analysed by LC-MS as described below.

2.14 Sample solubilisation and quantification

Protein pellets were solubilised by resuspending them in 50 mM triethylammonium bicarbonate (TEAB; Sigma), 2% Sodium dodecyl sulfate (SDS; Sigma) and placed at 95 ˚C for five minutes. Thereafter, samples were clarified by centrifugation at 10000 x *g* for five minutes. Quantification was performed using the QuantiPro BCA assay kit (Sigma) according to the manufacturer's instructions.

2.15 On-bead HILIC digest

In preparation for the hydrophilic interaction liquid chromatography (HILIC) magnetic bead workflow, beads were aliquoted into a sterile tube, and the shipping solution was removed. The beads were then washed with 250 µl wash buffer (15% ACN, 100 mM Ammonium acetate [pH 4.5]) for one minute. This was repeated once. The beads were then re-suspended in loading buffer (30% ACN, 200mM Ammonium acetate pH 4.5). The rest of the process described hereafter was performed using a Hamilton MassSTAR robotics liquid handler (Hamilton, Switzerland). A total of 50 µg of protein from each sample was transferred to a protein LoBind plate (Merck). Protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma) which was added to a final concentration of 10 mM TCEP and incubated at 60˚C for one hour. Samples were cooled to room temperature and then alkylated with 10 mM methylmethanethiosulphonate (MMTS; Sigma) and incubated at room temperature for 15 minutes. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on the shaker at 900 RPM for 30 minutes for binding of protein to beads. After binding, the beads were washed twice with 500 µl of 95% ACN for one minute. For digestion, trypsin (Promega) made up in 50mM TEAB was added at a ratio of 1:10 total protein, and the plate was incubated at 37˚C on the shaker for four hours. After digestion, the supernatant containing peptides was removed and dried down. Samples were resuspended in 0.1% trifluoroacetic acid (TFA; Sigma) prior to clean up by Zip-Tip (Sigma). Thereafter, samples were dried down once more and then resuspended in LC loading buffer: 0.1% FA, 2.5% ACN.

2.16 Liquid chromatography–mass spectrometry (LCMS)

LCMS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. Peptides were dissolved in 0.1% formic acid (FA; Sigma), 2% acetonitrile (ACN; Burdick & Jackson) and loaded on a C18 trap column (300 µm × 5 mm × 5 µm) at 3.5% solvent B and a flow rate of 5µl/min and washed for four minutes. Chromatographic separation was performed with a PepAcclaim C18 column (75 µm × 25 cm × 2 µm) as described below. The solvent

system employed was solvent A: LC water (Burdick and Jackson); 0.1% FA and solvent B: ACN, 0.1% FA. The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 6 min, gradient change: 3.5 – 9% Solvent B, time change 45.5 min, gradient change 9 – 24.6% Solvent B, time change 2 min, gradient change 24.6 – 38.7% Solvent B, time change 2.1 min, gradient change 38.7 – 52.8% Solvent B, time change 0.4 min, gradient change 52.8 – 85.4%. The gradient was then held at 85.4% solvent B for 10 minutes before returning it to 3.5% solvent B for 15 minutes to condition the column, resulting in a total of 81 minutes for each experiment. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 KV. Details of data acquisition are shown in the table below.

Table Details of data acquisition

Full Scan	
Resolution	70,000 (@ *m/z* 200)
AGC target value	1e6
Scan range	350-2000 m/z
Maximal injection time (ms)	250
Data-dependent MS/MS	
Inclusion	Off
Number of MS/MS scans produced	1,000,863
Resolution	17,500 (@ *m/z* 200)
AGC target value	1e5
Maximal injection time (ms)	50
Loop Count	5
Isolation window width (Da)	2
NCE (%)	27
Data-dependent Settings	
Underfill ratio (%)	1
Charge exclusion	1, 7,8, >8
Peptide match	Preferred
Exclusion isotopes	On
Dynamic exclusion (s)	60

2.17 Bioinformatics analysis

2.17.1 Source of data collection

A total of twenty 81-minute LCMS/MS experiments (5 biological replicates of each in maize and sorghum under both well-watered and water-deprived conditions) were acquired from the Thermo Q-exactive at the Centre for Proteomics and Genomics Research. Three additional

LC-MS/MS experiments represented super pools. On average across the two plant species, these files included approximately 30,000 MS/MS spectra each. Phytozome protein databases (Goodstein *et al*., 2001) downloaded October 11th, 2017 holds 88,760 proteins for *Z. mays* and 47,121 proteins for *S. bicolor*. For a further interpretation, proteins were then classified with the use of a Gene Ontology (GO) term enrichment approach.

2.17.2 Peptide and Protein identification pipeline

The raw data (spectra) acquired by the LC-MS/MS were converted to mzML format via ProteoWizard 3.0 msConvert (Kessner *et al*., 2008) using peakPicking and Zlib compression to provide input files for database searching.

Database search was achieved by MS-GF+ search engine (Jan. 13, 2017) (Kim and Pevzner., 2014) to determine the potential peptides, this was formed by semi-tryptic specificity and precursor tolerance of 20ppm was applied. Post-translational modifications (PTMs) were identified including, oxidation and carbamylation.

The data search results were processed by IDPicker 3.1 9729 (Ma *et al*., 2009) to yield a 5% PSM FDR, but required two distinct peptide sequences for each protein. Peptides passing these thresholds were deemed as genuine identifications.

NCBI BLAST 2.5.0+ makeblastdb (Boratyn *et al*., 2013) produced indexed versions of the FASTAs, which were used to identify orthologs (the same gene in two different species) between the two plant species. For each pair of databases (maize and sorghum), the blastp software searched for matches for each sequence of maize in sorghum and for each sequence of sorghum in maize using output format "6 qseqid sseqid length qstart qend pident bitscore evalue stitle". Orthologs tables generated were read in a script in the R statistical environment and applied a bitscore threshold of 50 to eliminate distant matches. When multiple matches detected, only the highest bitscore was retained.

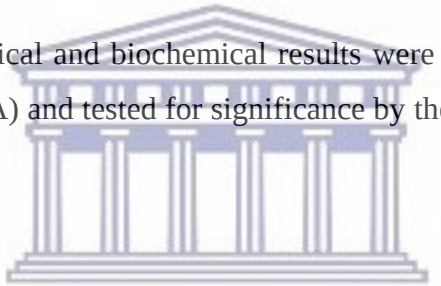
Spectral Count Rows were aligned by reading the orthology information and the spectral count table from IDPicker in a script in the R statistical environment. Accessions for each table row were analyzed to determine relationships to other rows. Proteins lacking orthologs or for which orthologs were not identified were excluded from further analysis. When maize and

sorghum orthologs were separated to different rows, the two rows were combined to make one joint row.

2.18 Statistical analysis

Based on five biological replicated on pairwise comparisons of a different cohort (well-watered and water deprived) were used, the protein identification false discovery rate was calculated as defined by Tabb (2007). For the spectral count data comparison, the input files were read in a script in R environment, applying minimum information criterion (MIC: 10 spectra per protein), and then a Quasi-Poisson model was constructed with species and (control/water-deprived) variables. The Benjamini-Hochberg FDR method (Benjamini *et al*., 2001) was performed to correct p-values for multiple testing. Proteins with a q-value < 0.05 are considered significantly different, it is expected that 5% of the claimed changes will be false positives.

For all other tests, physiological and biochemical results were analysed using the one-way analysis of variance (ANOVA) and tested for significance by the Tukey-Kramer test at a 5% level of significance.



Chapter Three

Results

3.1 Evaluation of the physiological and Biochemical Responses

3.1.1 Reduction in growth is more severe in *Zea mays* than in *Sorghum bicolor*

The effect of water deprivation on the growth of maize and sorghum was determined by observing the physiological responses including the shoot fresh weights (Figure 3.1 a), shoot length (Figure 3.1 b) and root length (Figure 3.1 c). The shoot fresh weights and shoot length were measured at the V8 stage of growth. Under drought, the shoot weights of maize were reduced by approximately 84 % when compared to the control plants. However, a 77 % decrease in fresh weights was observed for sorghum in response to water deprivation when compared to the well-watered plants. The shoot length of maize was decreased under water deficit by roughly 29 % compared to the respective control, while the length reduction on sorghum under drought stress was approximately 16 % compared to the control plants. In response to drought, sorghum plants displayed a 44 % increase in the root length, whereas maize showed only a 14 % increase in the root length when compared to the respective controls.

In addition, water deficit induced morphological changes in both maize and sorghum plants. The old leaves of drought-treated plants dried up and dropped off as a result of water stress. This took 40 days to occur after the last watering for maize whereas it took 55 days for sorghum. Water deprivation decreased the number of tillers produced in sorghum plants by approximately 70 % when compared to well-watered plants. Neither the maize nor sorghum plants reached the reproductive stage at the time of harvest.

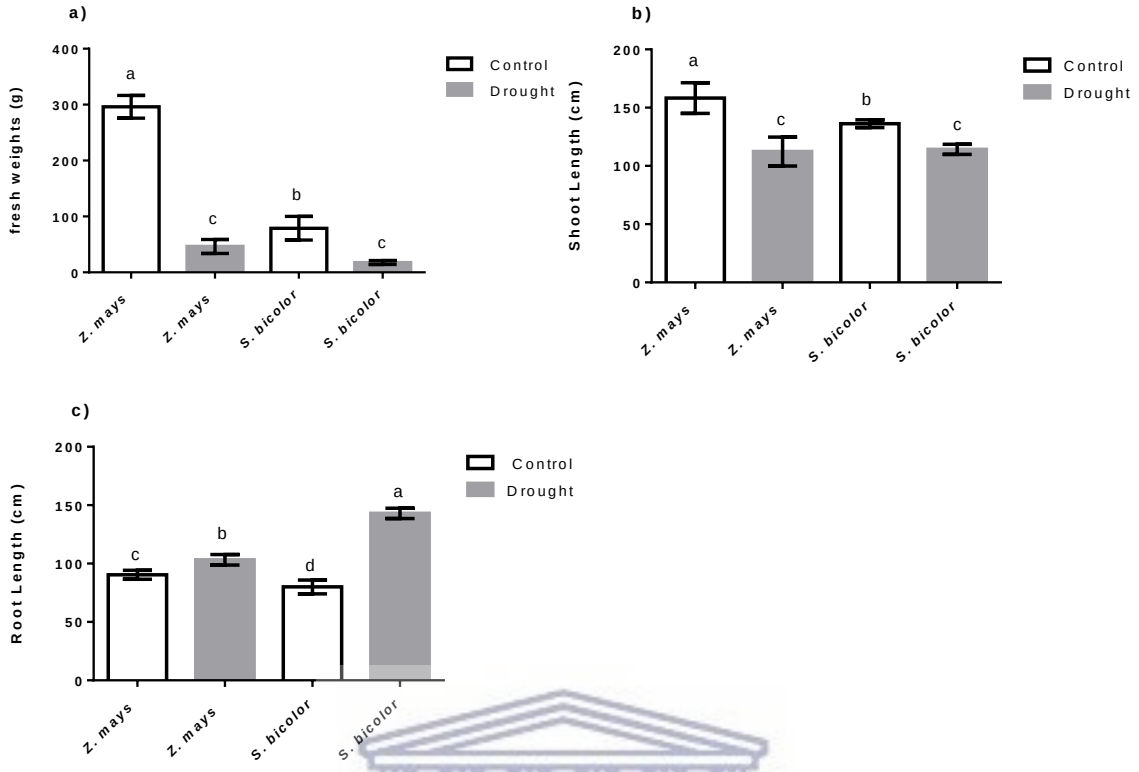


Figure 3.1 The effect of drought stress on shoot weights (a) shoot length (b) and root length (c) of *Zea mays* and *Sorghum bicolor*.

The stress parameters of well-watered and water-deprived plants were determined at V8 stage of growth. Values are means ± SE of 10 plants from ten independent experiments, $P \leq 0.05$.

3.1.2 Accumulation of proline in the roots of *Sorghum bicolor* improves water retention

The exposure of the two plant species to drought significantly influenced the relative water content and proline accumulation. The water status in plants is one of the most crucial stress parameters that indicate plant responses to drought. Drought stress reduced the relative water content of both plant species when compared to their well-watered plants (Fig. 3.2 c). However, maize plants exhibited a drastic decline of (±30%) water content while sorghum plants displayed only (±7%) decrease in water content under drought-induced conditions. The ability to retain water indicates the degree of tolerance to drought. In (figure 3.2 a) total proline content was shown to be considerably higher (±62.6% and ±49.6%) in the leaves of *Zea mays* and *Sorghum bicolor*, respectively. Interestingly, the total proline accumulation in the roots of sorghum was significantly enhanced under drought (±62.5%) while a smaller (±44.5%) increase was observed in the roots of maize when compared to the well-watered plants (Fig. 3.2 b).

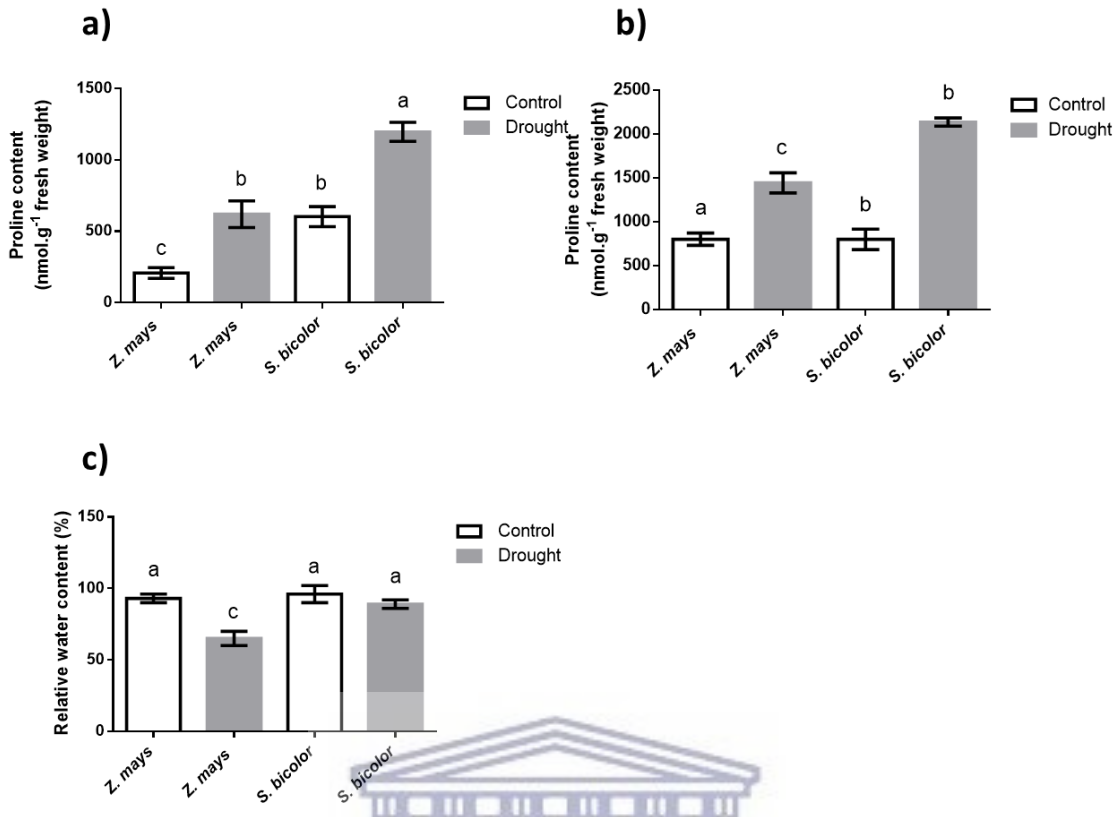


Figure 3.2 effect of drought on total proline accumulation (a and b) and water retention (c) in *Zea mays* and *Sorghum bicolor*.
Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P \leq 0.05$.

3.1.3 Drought reduces chlorophyll content in both *Zea mays* and *Sorghum bicolor* plants

Chlorophyll fluorescence (CF) is one of the most commonly used methods for measuring and in some cases categorizing a range of stresses that affect photosynthesis. It is used extensively to identify stress and plant responses to environmental changes (Banks, 2018). In the presence of water deprivation, both maize and sorghum showed a significant reduction in chlorophyll *a* and *b* content when compared to their respective controls. Maize plants subjected to drought stress showed a reduction of 26% in chlorophyll *a*, 26% in chlorophyll *b* and approximately 26% in total chlorophyll content. Drought reduced chlorophyll a, b and total chlorophyll

content in sorghum by 27%, 27% and 27% respectively when compared to well-watered plants.

Table 3-1 The effect of water deprivation on the chlorophyll content in two plant species.

	Chlorophyll a	Chlorophyll b	Total chlorophyll
WW *Z. mays*	0.6269 ± 0.0024[a]	1.155 ± 0.0045[a]	1.782 ± 0.0069[a]
WD *Z. mays*	0.4636 ± 0.0029[b]	0.852 ± 0.0055[b]	1.315 ± 0.0084[a]
WW *S. bicolor*	0.5433 ± 0.0076[a]	1.001 ± 0.0141[a]	1.544 ± 0.0217[b]
WD *S. bicolor*	0.3965 ± 0.0019[c]	0.729 ± 0.0035[c]	1.125 ± 0.0055[d]

Data presented are means ± SE; n= 3. Different letters indicate significant differences between means at $P \leq 0.05$. WW (Well-watered); WD (water deficit).

3.1.4 Oxidative stress is more pronounced in *Zea mays* than in *Sorghum bicolor* under water deprivation

Figure 3.5. (a-f) represents hydrogen peroxide content, superoxide content and lipid peroxidation level (MDA content) in leaves and roots of maize and sorghum plants grown in the presence and absence of water limitations. Drought stress triggered changes in hydrogen peroxide content in the leaves and roots of maize and sorghum (Figure 3.5. a and b). In the leaves (Figure 3.5. a), maize plants showed an approximately 70% increase in H_2O_2 content in the absence of sufficient water while sorghum plants exhibited a 55% increase in H_2O_2 content. However, there were no significant changes of H_2O_2 content in the roots of both species as shown in Figure 3.5. b. Figure 3.5. c shows that under water deprivation, $O_2^{\cdot}$ content was increased by approximately 38.6% in leaves of maize, while it remained unchanged in leaves of sorghum when compared to their respective controls. The trend was observed in the roots (Figure 3.5. d), in which an approximately 25% increase in $O_2^{\cdot}$ content was observed

in *Zea mays*, while no statistically significant change in $O_2^{\cdot}$ was observed in *Sorghum bicolor* when compared to the well-watered plants. The level of lipid peroxidation as estimated by MDA content in the leaves and roots of well-watered maize and sorghum as compared to water-deprived plants is shown in Figure 3.5. e and f, where the MDA content in the leaves (Figure 3.5. e) of water-deprived maize increased by approximately 58 % whereas MDA content was approximately 41 % higher in the leaves of water-deprived sorghum plants. The roots of maize and sorghum, as shown in Figure 3.5. f, had differing MDA content. A 24 % increase in MDA content was observed in water-deprived maize plants, while there was no significant change in MDA content was observed in water-deprived sorghum plants when compared to well-watered plants (Figure 3.5. f). The results obtained above indicate that the degree of oxidative damage in maize is more pronounced than in sorghum in response to drought.

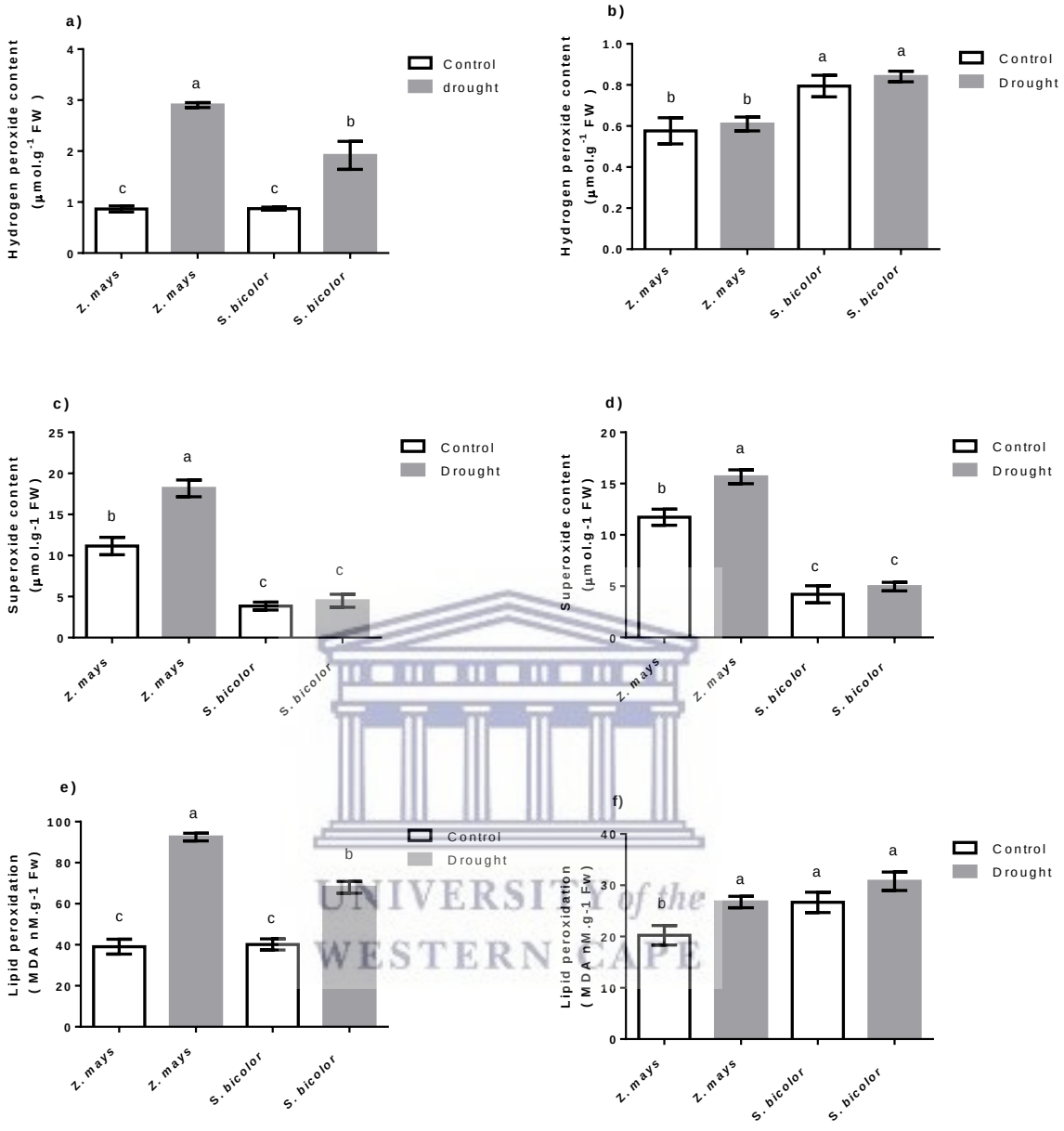


Figure 3.3 The degree of oxidative stress exhibited in *Zea mays* and *Sorghum bicolor* under well-watered and water-deprived conditions.

Hydrogen peroxide (H_2O_2) (a and b) in leaves and roots and $O_2^{\cdot}$ (c and d) in leaves and roots were measured as the indication of ROS accumulation, and lipid peroxidation (e and

f) in leaves and roots respectively was measured as the indication of oxidative stress. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P \leq 0.05$.

3.1.5 Drought stress induces cell death in *Zea mays* and *Sorghum bicolor*

Figure 3.3 illustrates the level of cell death as estimated by Evans Blue uptake. The levels of cell death for both the maize and sorghum leaves were higher under water deprivation when compared to their relative controls. Under drought stress, the level of cell death in maize was increased by approximately 12 % while cell death in sorghum was approximately 7 % greater when compared to their well-watered plants.

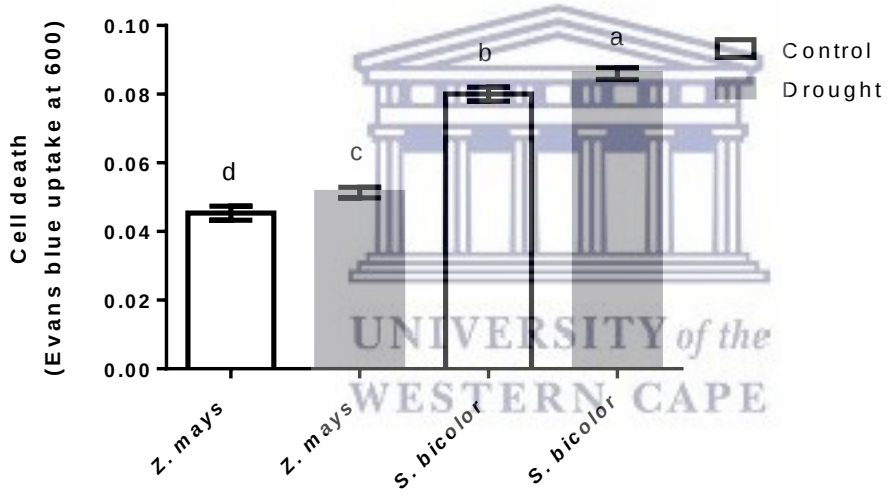


Figure 3.4 The effect of drought stress on cell viability in *Zea mays* and *Sorghum bicolor*.
Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P \leq 0.05$.

3.1.6 Effects of drought on superoxide dismutase (SOD) activity in the leaves and roots of *Zea mays* and *Sorghum bicolor*

The activity of SOD isozymes in the two plant species under water stress was determined using native PAGE gels (Figure 3.5), and the total SOD activity was also measured using a spectrophotometer-based assay (Figure 3.6). A total of six SOD isozymes were observed in the leaves and roots of maize and sorghum (Figure 3.5). The SOD isozymes were identified by using 5 mM KCN and 6 mM H_2O_2 as inhibiters. Isoforms that were unaffected by both H_2O_2 and KCN were identified as MnSOD whereas isoforms that were affected by both KCN and H_2O_2 were identified as FeSOD. Those affected by KCN alone were identified as Cu/ZnSODs. Based on these treatments, The SOD profile of maize and sorghum included two manganese superoxide dismutase (MnSOD), three copper/zinc superoxide dismutases (Cu/Zn SOD) and one iron superoxide dismutase (Fe-SOD) isoforms.

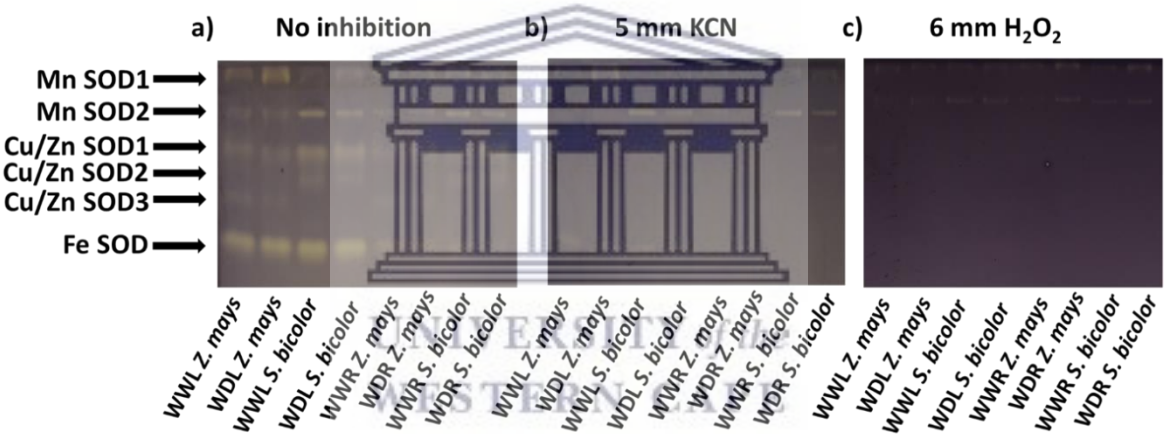


Figure 3.5 Changes on superoxide dismutase (SOD) isoenzymes activities in native gels in leaves and roots of *Zea mays* and *Sorghum bicolor* in response to drought stress.
The three in-gel assays represent the detection of different SOD isoforms (a) without inhibitors, (b) treated with 5 mM KCN and (c) treated with 6 mM H_2O_2. The WW represent well-watered, WD represents water-deprived, L represents the leaves and R represents the roots.

As shown in Figure 3.6, the total activity of SOD isozymes showed a slight increase

(approximately 12%) in activity in maize in response to drought stress when compared to their controls, while SOD total activity in sorghum remained unchanged under water-deprived conditions. However, a significant decrease in total SOD activity was observed in the roots of both maize and sorghum plants, (20% and 22%, respectively).

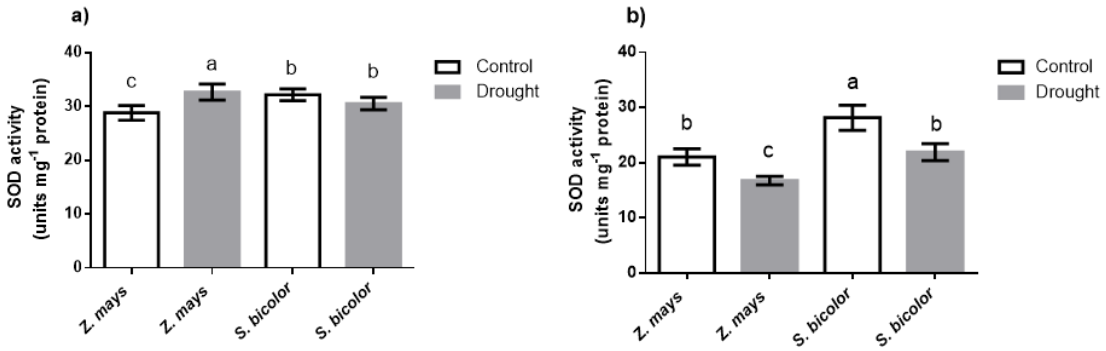


Figure 3.6 Spectrophotometric determination of the total SOD activity in the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*
Error bars represent the means ± SE; n= 3. Different letters indicate significant differences between means at $P \leq 0.05$.

Densitometry analysis was also performed using Alpha Ease FC Software to determine the pixel intensities in the individual SOD isoforms, as shown in Table 3-2. a and b. The densitometry results showed variation of SOD isoform activity between the two plant species. There was a significant increase in MnSOD 1 isoform activity under drought in the leaves of both maize and sorghum (45% and 30%, respectively), while a different trend was shown in the roots where a 20% decrease in maize and a 52% decrease in sorghum occurred when compared to their relative controls. The MnSOD 2 isoform was down-regulated by 10% in the leaves of maize but remained unchanged in the roots (Table 3-2). MnSOD2 was down-regulated by 37% and 14% in sorghum leaves and roots, respectively (Table 3-2). Cu/Zn-SOD 1 displayed a 22% increase in the leaves of maize and a 25% increase in sorghum leaves and was similarly increased in the roots of both plant species. Cu/Zn-SOD 2 activity was not

detectable in the leaves and roots of maize, while Cu/Zn-SOD 2 was decreased in the leaves of sorghum by 22% and no change was observed in sorghum roots. Even though Cu/Zn-SOD 3 was only present in the maize plants, the activity in the both leaves and roots was significantly downregulated in response to water deprivation. A slight decrease (approximately 12%) in Fe-SOD isoform activity was observed in the leaves of maize plants, whereas no significant change occurred in the leaves of sorghum when compared to the control plants for this isoform. However, both plant species showed a similar trend in Fe-SOD isoform activity in the roots, where a 34% decrease was observed.

Table 3-2 Densitometry readings for SOD native PAGE activity gels of the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*. (figure 3.5).

a)

SOD isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Mn SOD1	1±0,00^{c}	1.820±0.050^{a}	1±0,00^{c}	1.435±0,036^{b}
Mn SOD2	1±0.00^{a}	0.899±0,006^{b}	1±0.00^{a}	0.632±0,017^{b}
Cu/Zn SOD1	1±0,00^{c}	1.224±0,014^{b}	1±0,00^{c}	1.247±0,065^{a}
Cu/Zn SOD2	NA	NA	1±0,00^{a}	0.776±0,014^{b}
Cu/Zn SOD3	1±0,00^{a}	0.776±0,014^{b}	NA	NA
Fe SOD	1±0.00^{a}	0.884±0.004^{b}	1±0.00^{a}	1.002±.041^{a}

b)

SOD isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Mn SOD1	1±0,00^{a}	0,799±0,012^{b}	1±0.00^{a}	0,478±0,036^{c}
Mn SOD2	1±0,00^{a}	1±0,05^{a}	1±0,00^{a}	0,857±0,006^{b}
Cu/Zn SOD1	1±0,00^{c}	1,302±0,018^{a}	1±0,00^{c}	1,251±0,012^{b}
Cu/Zn SOD2	NA	NA	1±0,00^{a}	1±0,05^{a}
Cu/Zn SOD3	1±0,00^{a}	0,747±0,018^{b}	NA	NA
Fe SOD	1±0,00^{a}	0.665±0,016^{b}	1±0,00^{a}	0.665±0,016^{b}

The letters WW represent well-watered, WD represents water-deprived and NA indicate that very low or no activity was detected. The relative pixel intensity values were measured using the Alpha Ease FC software and the SOD activities are expressed as arbitrary units, all SOD isoforms were normalized using the control of both plant species. Different letters indicate significant difference between means at $P \leq 0.05$ (DMRT). Values are means±S.E (n=3).

3.1.7 Drought stress significantly alters the ascorbate peroxidase activity in the leaves and roots of *Zea mays* and *Sorghum bicolor*

In this study, changes in ascorbate peroxidase enzyme activity in the leaves and roots of maize and sorghum were investigated under water deprivation. APX activity was determined by both native-PAGE and spectrophotometric assay. According to the native-PAGE analysis, four

APX isozymes were identified in the leaves and roots of maize while only three APX isoforms were detected in sorghum (Figure 3.7). The isoforms were named APX 1 to 3 in leaves and APX 1 to 4 in roots on the basis of their position in the gel. It is important to note that the identities of the APX isoforms are not known and that the names in leaves do not necessarily imply the same identity of an isoform in the roots even if they are referred to with the same name. Therefore, APX 1 in leaves is not necessarily the same APX as APX 1 in roots, and so on. Drought stress significantly altered the ascorbate peroxidase activity between the two plant species.

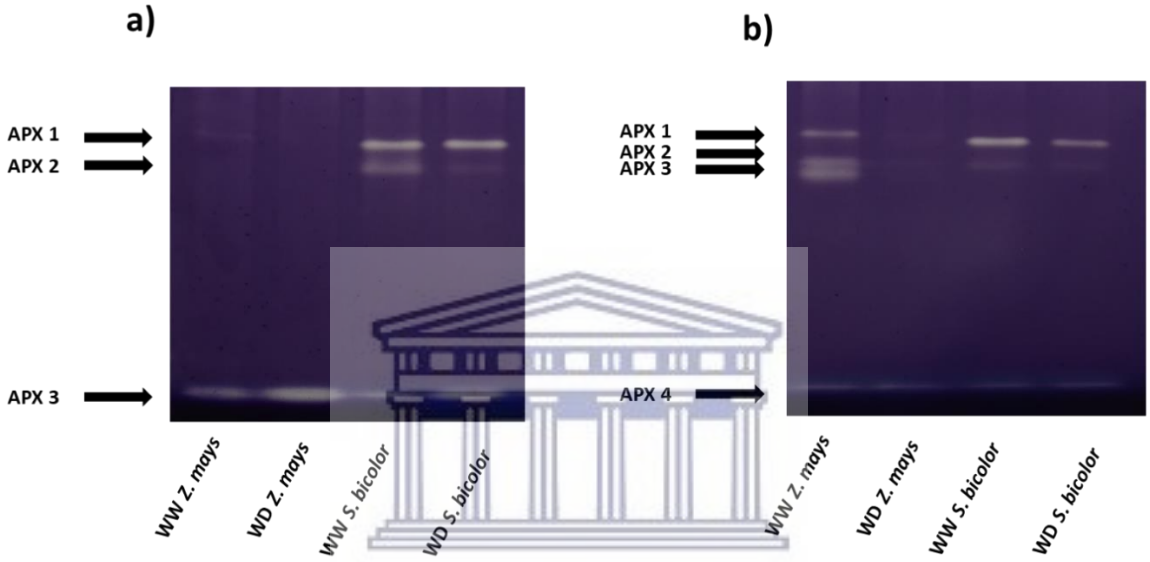


Figure 3.7 Changes in ascorbate peroxidase (APX) isoforms in response to drought stress in the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*.
WW represents well-watered, WD represents water-deprived.

Interestingly, the total APX activity measurements revealed that APX activity was significantly increased by approximately 38% in the leaves of *Zea mays* and by approximately 19% in the leaves of *Sorghum bicolor* (Figure 3.8 a). However, a pronounced decrease in APX total activity (by approximately 73%) was observed in the roots of *Zea mays*, while the roots of *Sorghum bicolor* showed no statistically different total APX activity under water deprivation when compared to the respective controls (Figure 3.8 b).

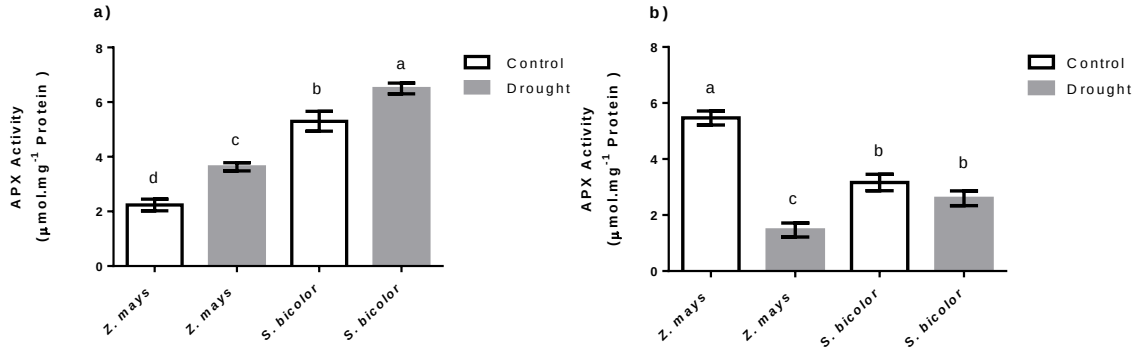


Figure 3.8 Determination of the total APX activity in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* by spectrophotometry.
Error bars represent the means ± SE; n= 3. Different letters indicate statistically different means ($P \leq 0.05$).

Based on the densitometry analysis (Table 3-3), it is observed that water deprivation significantly altered APX isoform activity in the leaves of both *Zea mays* and *Sorghum bicolor*. Under water deprivation, APX1 isoform activity was significantly decreased in the leaves of *Zea mays* (by approximately 46%) while it remained unchanged in the leaves of *Sorghum bicolor*. There was very low activity of the APX 2 isoform in the leaves of *Zea mays* and approximately 60% reduction in the activity of this isoform in the leaves of *Sorghum bicolor*. The densitometry analysis for the APX 3 isoform revealed that there was a significant increase in its activity in the leaves of *Zea mays* (approximately 77%), with an even higher increase (approximately 96%) observed for *Sorghum bicolor* when compared to the well-watered plants. The root analysis showed that APX 1 and APX 2 isoform activity was decreased in *Zea mays* (approximately 68% and 79%, respectively), with a lower extent of decrease in *Sorghum bicolor* (44% and 18%, respectively). The analysis also showed that the activity of the APX 3 isoform was significantly increased (by approximately 84%) in *Zea mays* when compared to the control plants, while no activity was detected in both control and water-deprived *Sorghum bicolor* plants. However, the APX 4 isoform showed no significant changes in the roots of *Zea mays*, while an increase of approximately 22% was observed in the roots of water-deprived *Sorghum bicolor* when compared to the well-watered plants.

Table 3-3 Densitometry readings for APX isoforms in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* as observed on native acrylamide gel (Figure 3.7).

a)

APX isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Isoform 1	1±0,00[a]	0,543±0,024[b]	1±0.00[a]	0,988±0,006[a]
Isoform 2	NA	NA	1±0.00[a]	0.4±0,004[b]
Isoform 3	1±0.00[c]	1.770±0.011[b]	1±0.00[c]	1.961±0.037[a]

b)

APX isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Isoform 1	1±0.00[a]	0.317±0,015[c]	1±0.00[a]	0.555±0.012[b]
Isoform 2	1±0.00[a]	0.206±0,014[d]	1±0.00[a]	0.817±0,010[c]
Isoform 3	1±0.00[a]	0.162±0,011[b]	NA	NA
Isoform 4	1±0.00[a]	0.904±.004[a]	1±0,00[a]	1.217±0,048[b]

The relative pixel intensity ratios were analyzed using the Alpha Ease FC software and the APX activities are expressed as arbitrary units, all APX isoforms were normalized using the control of both plant species. The letters WW represent well-watered, WD represents water-deprived and NA indicate that very low or no activity was detected. Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance.

3.1.8 Drought stress decreases catalase activity in maize and sorghum

Changes in catalase isozyme activity in *Zea mays* and *Sorghum bicolor* were investigated through the use of in-gel based and spectrophotometric assay. The native PAGE analysis revealed three CAT isoforms in the leaves of *Zea mays* and *Sorghum bicolor*. Only one CAT isoform was detected in the roots of *Zea mays*, while two CAT isoforms were detected in the

roots of *Sorghum bicolor*. In response to drought stress, the activity of all catalase isozymes was down-regulated in both *Zea mays* and *Sorghum bicolor* plants (Figure 3.9).

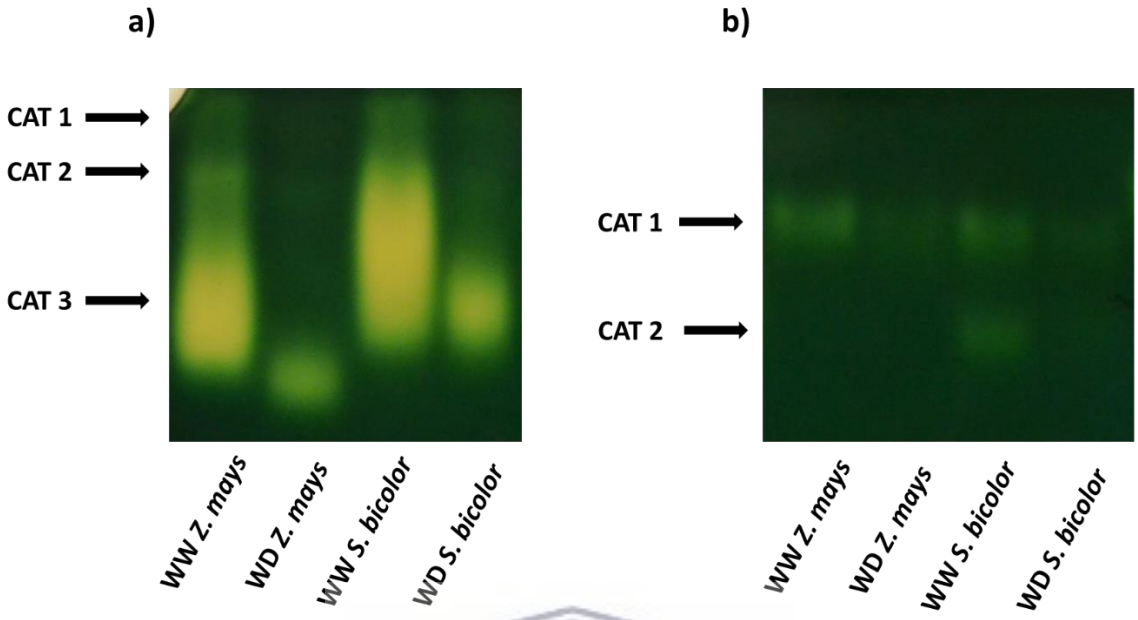


Figure 3.9 Catalase isoforms activity was decreased under water stress in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*.
The WW represent well-watered, WD represents water-deprived.

A spectrophotometric measurement of the catalase enzymatic activity was also conducted to determine the total activity of catalase isozymes (Figure 3.10). Drought stress inhibited the catalase total activity in the leaves of *Zea mays* by approximately 45% and by 33% in *Sorghum bicolor* plants in comparison to their relative controls. Similarly, a decrease of approximately 41% and 66% was observed in the roots of water-deprived *Zea mays* and *Sorghum bicolor* plants respectively when compared to their corresponding controls.

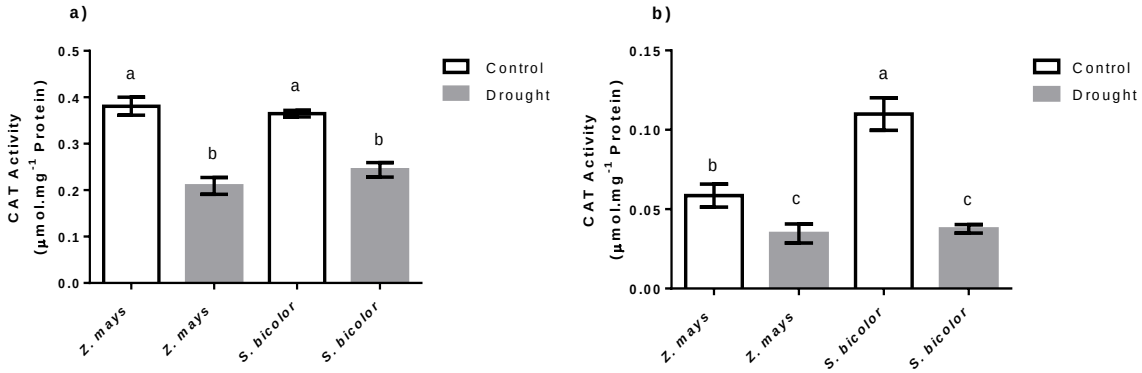


Figure 3.10 Determination of the total catalase activity in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* by spectrophotometry assay.
Error bars represent the means ± SE; n= 3. Different letters indicate statistically different means ($P \leq 0.05$).

The relative pixel intensity ratio was also measured to determine the changes in individual CAT isoforms as shown in (Table 3-4). Under water deprivation, all CAT isoforms were significantly down-regulated in the leaves of both *Zea mays* and *Sorghum bicolor* plants in comparison to their respective controls (Table 3-4 a). However, the same trend was observed in the roots of both *Zea mays* and *Sorghum bicolor* when subjected to water deprivation (Table 3-4 b).

Table 3-4 Densitometry readings for CAT isoforms in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* as observed on native acrylamide gel (Figure 3.9).

a)

CAT isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Isoform 1	1±0.00^{a}	0.610±0.013^{c}	1±0.00^{a}	0.637±0.015^{b}
Isoform 2	1±0.00^{a}	0.414±0.011^{b}	1±0.00^{a}	0.410±0,008^{c}
Isoform 3	1±0.00^{a}	0.606±0.006^{c}	1±0.00^{a}	0.728±0.003^{b}

b)

CAT Isoforms	Plant species/treatment			
	WW *Z. mays*	WD *Z. mays*	WW *S. bicolor*	WD *S. bicolor*
Isoform 1	1±0.00^{a}	0.615±0,047^{b}	1±0.00^{a}	0.572±0,015^{c}
Isoform 2	NA	NA	1±0.00^{a}	NA

The pixel intensity values were measured using the Alpha Ease FC software and the CAT activities are expressed as arbitrary units, all CAT isoforms were normalized using the control of both plant species. The letters WW represent well-watered, WD represents water-deprived and NA shows that very little or no activity was observed. Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance.

3.1.9 Glutathione reductase in *Zea mays* and *Sorghum bicolor* is differentially regulated in response to water deprivation

The GR native-PAGE activity gel displayed six isoforms present in *Zea mays* and *Sorghum bicolor*. As a result of plant exposure to water deprivation, glutathione reductase isoforms were differentially regulated as shown in Figure 3.11.

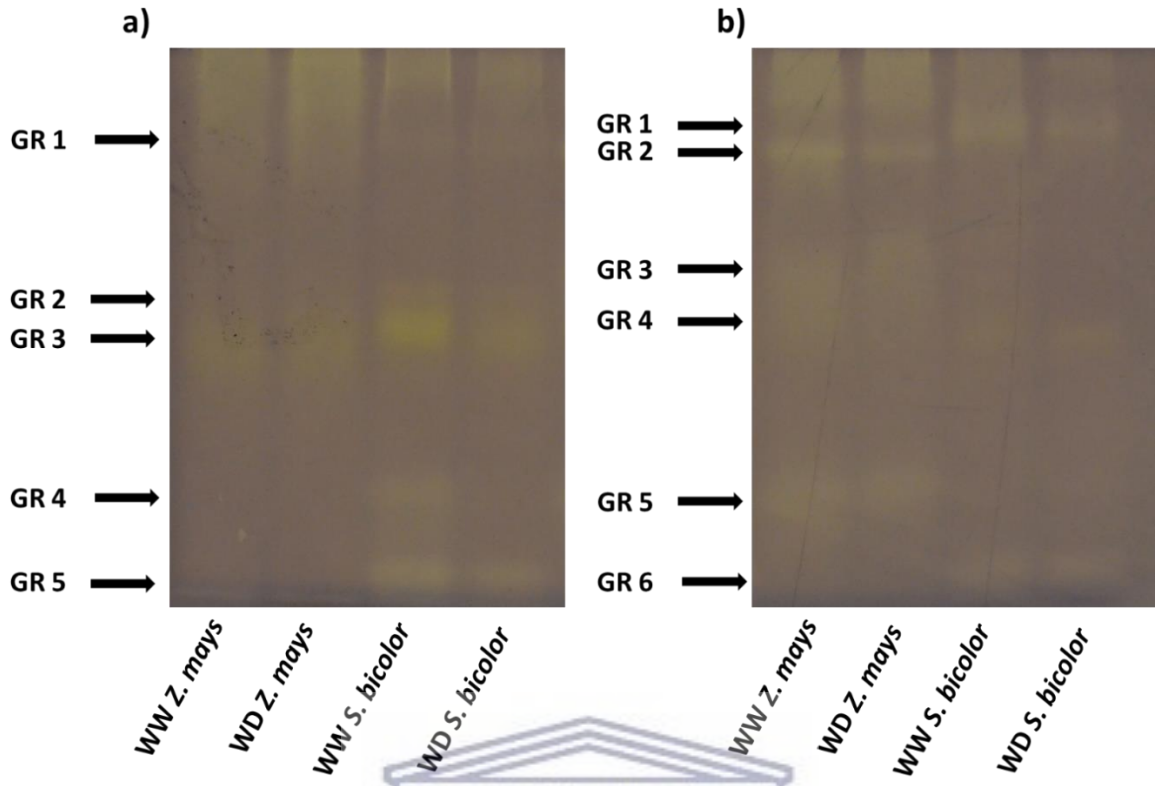


Figure 3.11 Changes on glutathione reductase (GR) isozymes activity in native gels in leaves and roots of two plant species in response to drought stress.
The WW represent well-watered, WD represents water-deprived, L represents the leaves and R represents the roots.

Spectrophotometric results for total GR activity displayed an approximately 33% increase in the leaves of *Zea mays* under drought, while no change was observed in the leaves of *Sorghum bicolor* when compared to the well-watered plants (Figure 3.12 a). However, glutathione reductase activity was down-regulated in the roots of *Zea mays* by approximately 16%, whereas the GR activity was approximately 22% higher in the roots of *Sorghum bicolor* that were exposed to water deprivation when compared to their respective controls (Figure 3.12 b).

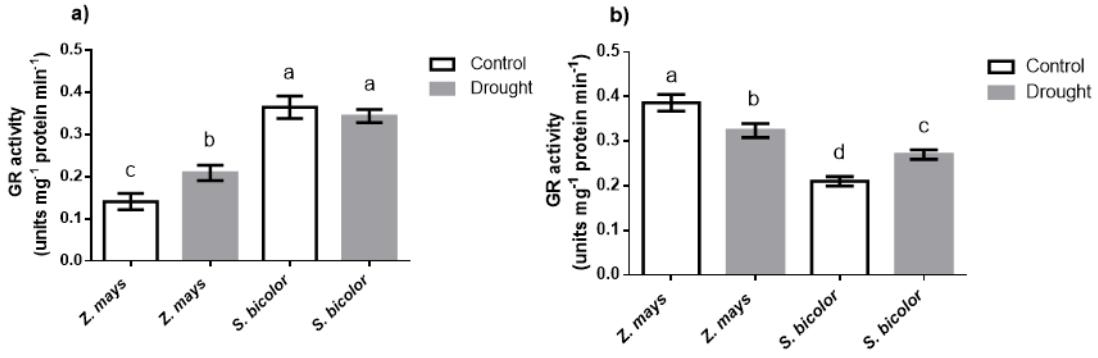


Figure 3.12 Determination of the total glutathione reductase activity in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* by spectrophotometry.
Error bars represent the means ± SE; n= 3. Different letters indicate statistically different means ($P \leq 0.05$).

Interestingly the relative pixel intensity ratio as shown in (Table 3-5 a) showed that GR 1, GR 2, GR 4 and GR 5 isoforms were only present in the leaves of *sorghum biocolor*. However, GR 1 activity was up-regulated by approximately 22% when these plants were deprived of water. The activity of GR 3, GR 4 and GR 5 was significantly inhibited by approximately 12%, 29.4% and 22% respectively, while GR 2 activity remained unchanged. An approximately 33% increase in the GR 3 isoform activity was observed in the leaves of *Zea mays* in response to water deprivation when compared to the control plants. As shown in Table 3-5 b, the GR 1 and GR 6 isoform activities were only detected in the roots of *Sorghum bicolor*, the former showed a decrease of 16%, while the later displayed an increase of 29% in response to drought. In contrast, GR 2, GR 3 and GR 5 isoforms were only present in the roots of *Zea mays*. Their activity was significantly inhibited by water deprivation when compared to their respective controls. Lastly, the results also showed that under water-deprived conditions, the GR 4 isoform activity was decreased by approximately 25% in the roots of *Zea mays*, while no difference was observed in *Sorghum bicolor* when compared to the well-watered plants.

Table 3-5 Densitometry readings for glutathione reductase (GR) isoforms in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* as observed on native acrylamide gel (Figure 3.12).

a)

GR Isoforms	Plant species/treatment			
	WW *Z. mays*	**WD *Z. mays***	**WW *S. bicolor***	**WD *S. bicolor***
Isoform 1	NA	NA	1±0,00[b]	1,215±0,009[a]
Isoform 2	NA	NA	1±0,00[a]	0,935±0,080[a]
Isoform 3	1±0,00[b]	1,333±0,049[a]	1±0,00[a]	0,880±0,008[b]
Isoform 4	NA	NA	1±0,00[a]	0.706±0,002[b]
Isoform 5	NA	NA	1±0,00[a]	0,780±0,051[b]

b)

GR Isoforms	Plant species/treatment			
	WW *Z. mays*	**WD *Z. mays***	**WW *S. bicolor***	**WD *S. bicolor***
Isoform 1	NA	NA	1±0,00[a]	0,840±0,009[b]
Isoform 2	1±0,00[a]	0,889±0,004[b]	NA	NA
Isoform 3	1±0,00[a]	0,944±0,002[b]	NA	NA
Isoform 4	1±0,00[a]	0,754±0,047[b]	1±0,00[a]	1,004±0,065[a]
Isoform 5	1±0,00[a]	1,137±0,048[b]	NA	NA
Isoform 6	NA	NA	1±0,00[b]	1,217±0,051[a]

The pixel intensity values were measured using the Alpha Ease FC software and the GR activities are expressed as arbitrary units, all GR isoforms were normalized using the control of both plant species. The letters WW represent well-watered, WD represent water-deprived and NA shows that very little or no activity was observed. Data presented in this table are the means ± standard error of three replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate significant difference between treatments at 5% level of significance.

3.2 Drought-responsive leaf proteins of *Zea mays* and *Sorghum bicolor* revealed by proteomics

3.2.1 One-dimensional protein electrophoresis of *Zea mays* and *Sorghum bicolor* leaf tissue

SDS-PAGE was conducted to evaluate the quality of the extracted protein prior to label-free mass spectrometry analysis. Coomassie Brilliant Blue (CBB)-stained leaf protein bands (in five replicates) of *Zea mays* and *Sorghum bicolor* were visible (Figure 3.13). Lane M represents the molecular weight marker. Lanes 1-10 represent protein profiles from five independent biological replicates for the leaf samples for each plant species. Each lane was loaded with approximately 10 µg of total protein extract (Figure 3.13). The results obtained from 1-D SDS-PAGE show that the quality of the leaf extracts was of decent quality, where no visible signs of streaking and protein degradation.

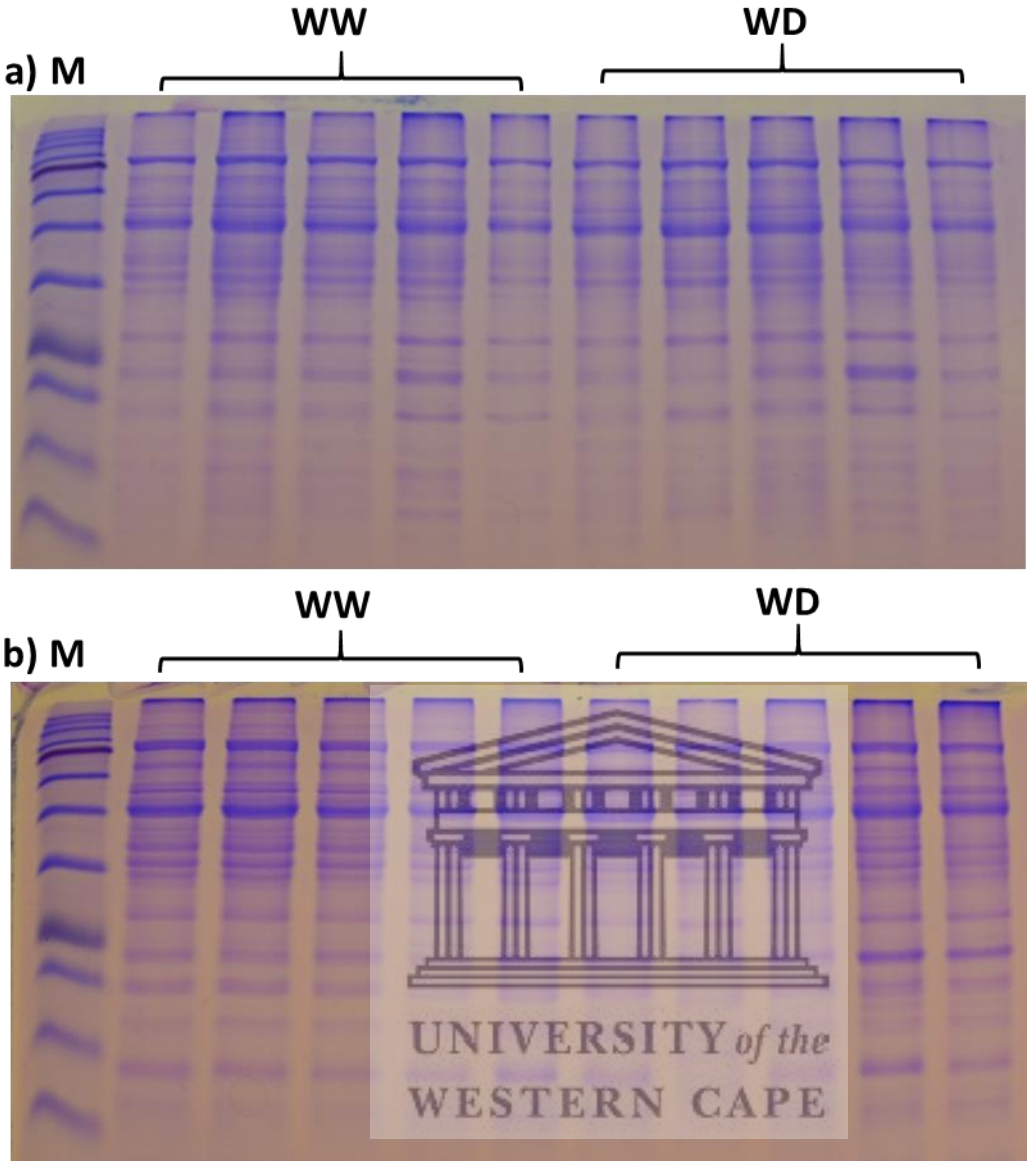


Figure 3.13 One-dimensional leaf proteins of *Zea mays* (a) and *Sorghum bicolor* (b) from five biological replicates.
Total soluble protein (10 µg) of leaf tissue for each plant species was loaded onto 12 % SDS-PAGE gels. Lane M is the molecular weight marker. Well-watered (WW), water-deprived (WD.

3.2.2 Identification of differentially expressed proteins between *Zea mays* and *Sorghum bicolor* in response to drought stress

Approximately 3154 detectable peptides were obtained in the spectral count table, of which 2752 entries contained a maize match and 2794 contained a sorghum match. However, 718 (23%) did not contain a match in either maize nor sorghum for which an ortholog had been named. 945 protein peptides (30%) contained a protein named in the ortholog map. However, the accession of its partner protein was not aligned to any identified protein group. Furthermore, 416 protein groups (13%) contained orthologs of both maize and sorghum. Among the peptides, 1070 peptides (34%) contained a protein for which the other ortholog was also identified. From those 1070 peptides, 535 composite rows were made, which coupled the maize spectrums from one of these rows and the spectrums of sorghum from another row. Orthology data has increased the number of proteins for which we could match maize and sorghum information from 416 to 951 different proteins (+ 129%). Quasi-Poisson analysis (minimum of 10 spectral counts at a q-value < 0.05) revealed that 207 orthologous protein groups were differentially expressed between maize and sorghum. Of the 207 orthologous groups of proteins, eight protein groups were identified to be differentially between well-watered and water-deprived plants between maize and sorghum, as shown in appendix (Table 3.6) with a grey highlight colour. Interestingly, among the 207 identified proteins, 88 proteins were over-represented in maize leaves exposed to water stress, whereas 106 proteins were over-represented in the leaves of water-stressed sorghum. However, 102 were suppressed in maize in response to water deficit whereas, only 92 proteins showed a decrease in their abundance in sorghum exposed to water deprivation (Figure 3.14).

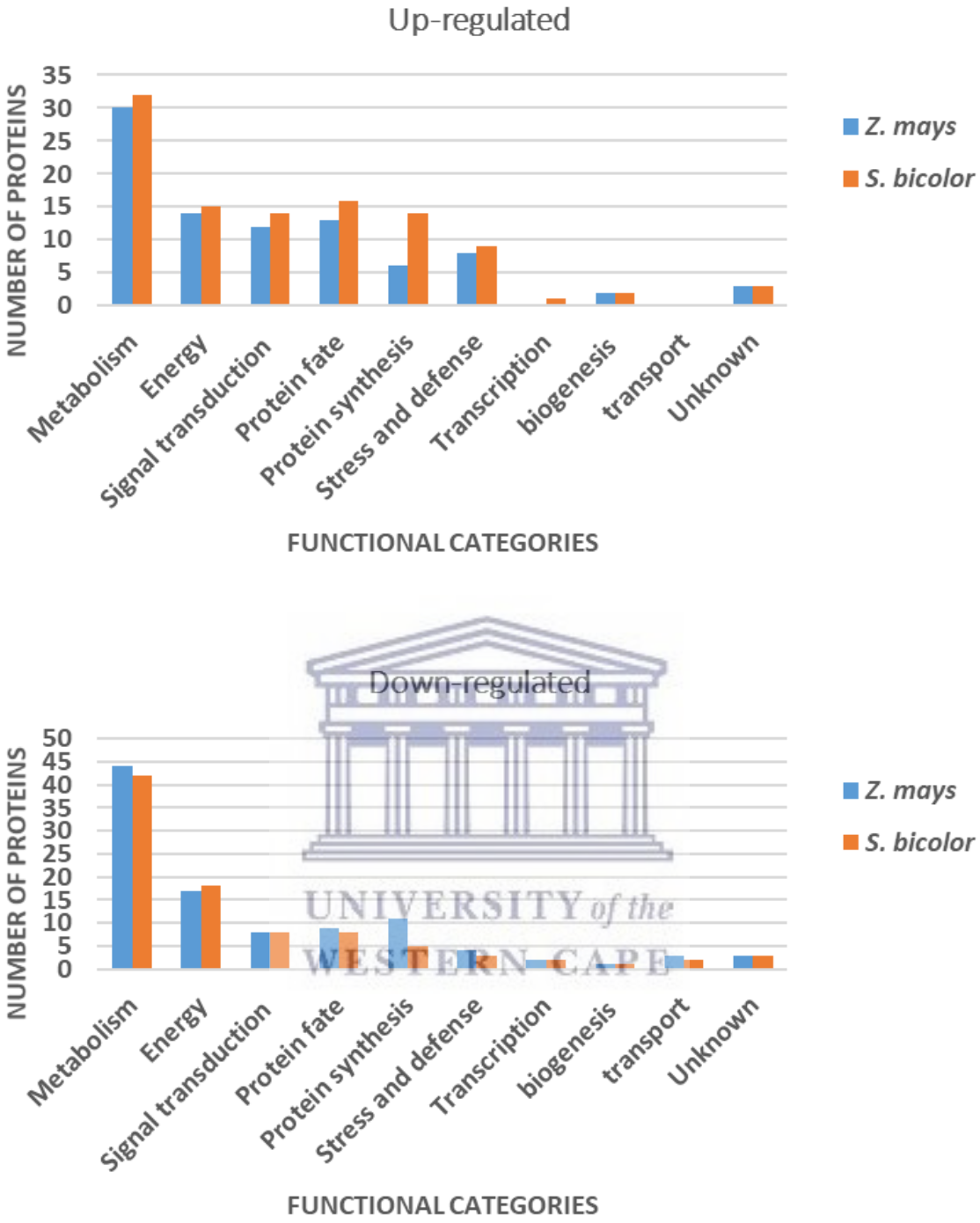


Figure 3.14 Differences in the expression (upregulation and downregulation) of functionally classified protein groups between *Z. mays* and *S. bicolor* under water stress.

3.2.3 Ontological characterization of differentially expressed proteins in maize and sorghum in response to water deprivation

Characterization of orthologous protein groups that showed significant differences in abundance in maize and sorghum (Table 3.6) were further investigated using a combination of similarity searches on OrthoDB (https://www.orthodb.org), UniProtKB (https://www.uniprot.org/help/uniprotk), OMA orthology database (https://omabrowser.org/oma/home/) and literature sources. The functional categories of the identified proteins were studied (Figure 3.15) and revealed that a large proportion (38.2%) of these proteins are involved in metabolism. The second largest group of proteins (16.4%) functionally associated to energy. Other functional categories included proteins involved in signal transduction mechanism (10.6%), protein fate (folding, modification, destination) (11.5%), protein synthesis (9.7%), stress and defence (5.8%), transcription (1.5%), Biogenesis of cellular component (1.5%), Transport and cell structure (1.5%). Others (3.4%) of the identified proteins were of unknown function.

The subcellular localization of a protein provides clues about its physiological function. Thus, the cellular components in which the identified proteins may occur were predicted using a combination of similarity searches on OrthoDB (https://www.orthodb.org), UniProtKB (https://www.uniprot.org/help/uniprotk), OMA orthology database (https://omabrowser.org/oma/home/), WoLF PSORT (https://wolfpsort.hgc.jp) and literature sources (Figure 3.16). Results showed that the identified proteins were mostly located in chloroplast (30.0%), cytoplasm (20.3%), membrane (9.2%), transmembrane (6.8%), cytosol (4.4%), nucleus (5.8%), extracellular matrix (4.3%), mitochondria (3.9%), small fractions of other subcellular localizations compromising the golgi apparatus, cell wall, amyloplast, peroxisome, vacuole, thylakoid membrane, myosin complex, proteasome core complex, ribosome, and chromoplast (15.5%) in total.

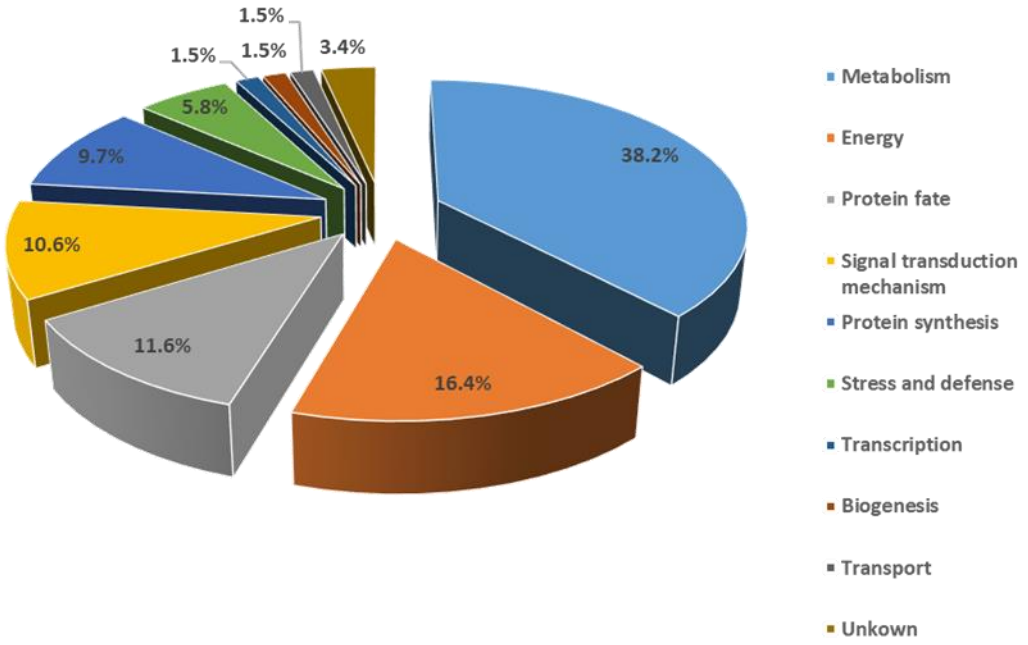


Figure 3.15 Functional classification of the 207 identified proteins in *Z. mays* and *S. bicolor* leaves

Functional categories were assigned using a combination of OrthoDB, UniProtKB, OMA orthology database and literature sources.

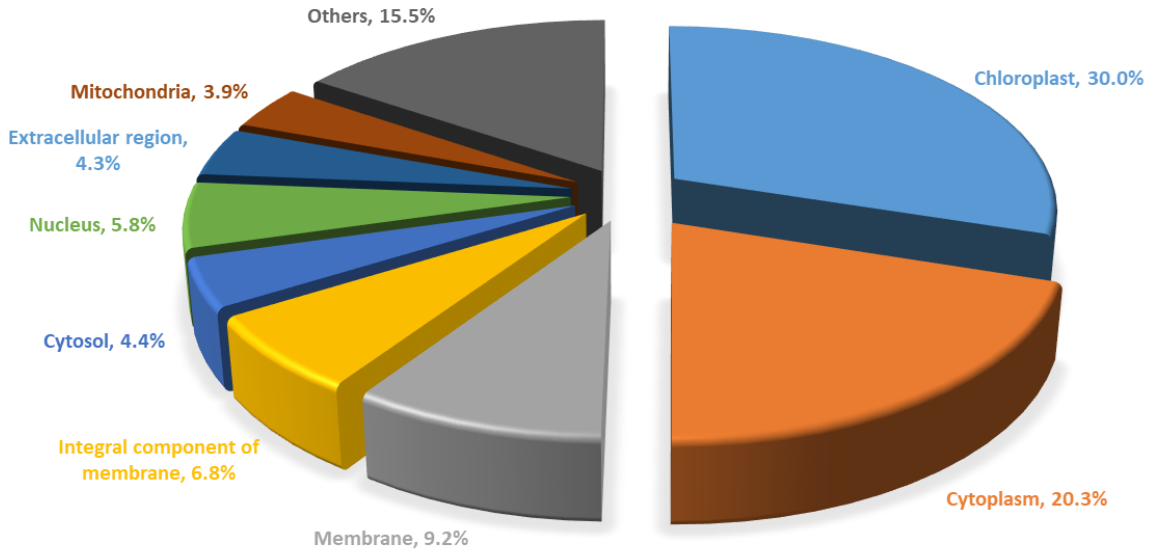


Figure 3.16 Subcellular localizations of identified maize and sorghum leaves. Subcellular localizations were predicted using a combination of OrthoDB, UniProtKB, OMA orthology database, WoLF PSORT and literature sources.

Chapter Four

Discussion

4.1 Evaluation of the physiological and biochemical responses

4.1.1 Drought stress alters physiological responses in *Zea mays* and *Sorghum bicolor*

As one of the most serious environmental stress for plants, drought adversely affects plant growth and development, limiting crop production more than any other environmental factor (Farooq *et al*., 2012). Both elongation and expansion of cell growth are influenced in response to drought stress (Anjum *et al*., 2011).

Several studies showed that, the exposure of crops to drought can result in reduced growth and limited yield (Jaleel *et al*., 2009; Barber *et al*., 2000; Skirycz and Inzé, 2010). Among abiotic stresses, drought is the main factor that limits maize production in many parts of the world, with significant yield loss being evident when maize experiences water stress around the flowering stage (Boyer and Westgate, 2004).

A similar trend was observed in this study. Under water deficit, *Zea mays* suffers a greater reduction of shoot weights and length than *Sorghum biocolor*. However, both plant species displayed a significant increase in their root length. The increase in root length was even greater in sorghum than in maize, similarly to the work reported by Wright *et al*. (1983). This increase of root length is likely an adaptive response to insufficient water availability. The ability of sorghum to form roots that are much longer than maize roots under drought stress may prove advantageous to sorghum in terms of its ability to reach deeper into soil to acquire moisture.

In cereal crops, deep roots are associated with a limited number of adventitious root, resulting in reduction of tillering (Blum, 2005). This study also revealed the same relationship between root length increase and tiller production in sorghum plants when exposed to water deprivation.

4.1.2 Accumulation of proline in the roots of *sorghum bicolor* improves water retention

Compatible osmolytes are powerful cytoprotectants that play a key role in plants against the effects of osmotic stress induced by water deficit stress (Yoshiba *et al.*, 1997). Proline is the most common organic osmolyte in water-stressed plants (Gomes *et al.*, 2010). Therefore, increased concentration of proline is associated with improved water retention capability (Quilambo, 2004). These reports are in agreement with the observations of this study, which confirmed that water deprivation enhanced the proline accumulation in the leaves and roots of maize and sorghum. Even so, when comparing the two species the increase in root proline content was higher in *Sorghum bicolor* than in *Zea mays*.

4.1.3 Drought decreases the chlorophyll content in both *Zea mays* and *Sorghum bicolor* plants

Photosynthetic pigments are considered essential for plant capacity to harvest light for photosynthesis. Water stress affects chlorophyll *a* and *b* (Chl a/b) synthesis and reduces the abundance of Chl a/b binding proteins, which leads to a decrease in the light-harvesting pigment associated with photosynthetic system II (Sayed, 2003). Drought effects on chlorophyll content were reported in sorghum (Masojídek *et al*., 1991), maize (Jovanovic *et al*., 1991), cotton (Mssacci *et al*., 2008) and *Catharanthus roseus* (Jaleel *et al*., 2008). In this study, differences in total chlorophyll content, chlorophyll *a* and chlorophyll *b* in the leaves of drought-stressed *Zea mays* and *Sorghum bicolor* were observed. Under limited water supply, *Zea mays* and *Sorghum bicolor* showed similar patterns: decreases in total leaf chlorophyll, chlorophyll a and chlorophyll b in response to drought. These results are not consistent with several studies (Pastori and Trippi, 1992; Kraus *et al*., 1995; Sairam, 1994) which demonstrate that high chlorophyll content is correlated with plant tolerance to water stress. However, a decrease of total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the light-absorbing pigments (Vanisri *et al.*, 2017). This conclusion is in agreement with the study by Herbinger *et al*. (2002) who described a significant decrease of chlorophyll a and b

caused by water deficit in two contrasting cultivars of wheat.

4.1.4 Drought stress induces cell death in *Zea mays* and *Sorghum bicolor*

Water deficit affects photosynthesis and increases photorespiration, alters normal cell homeostasis and leads to overproduction of reactive oxygen species (ROS) (Miller *et al*., 2010). This excessive production of ROS my cause severe oxidative damage and ultimately cell death (Sharma *et al*., 2012). However, effective ROS detoxification mechanisms and reduced cell death is associated with plant tolerance to water deficit (Genet *et al*. 2010).

Under water stress conditions described in this study, maize and sorghum showed increased level of cell death in comparison to their controls. Cell death increase was more pronounced in *Zea mays* when compared to *Sorghum bicolor*. The observed increase in cell death could be attributed by the higher level of MDA content in maize compared to sorghum (Figure 4.4 e and f). These results were consistent with previous studies (Keyster *et al*., 2013; Egbichi *et al*., 2014), where increased levels of MDA were accompanied by increased levels of cell death.

4.1.5 Oxidative stress is highly induced in *Zea mays* than *Sorghum bicolor* under water deprivation

Oxidative stress arises when the critical balance between ROS production and their scavenging by antioxidant enzymes is disrupted due to reduction of antioxidant enzymes or excess production of ROS, or both (Munné-Bosch and Peñuelas, 2004). This excessive accumulation of ROS is caused by variety of environmental stress conditions such as drought (Bartoli *et al.*, 1999). In this study, drought triggered accumulation of H_2O_2 in the leaves of maize and sorghum. The increase was more pronounced in *Zea mays* than in *Sorghum bicolor*. Drought stress increased superoxide content in *Zea mays* more than in *Sorghum bicolor*. The increased H_2O_2 and $O_2^{\cdot}$ contents observed here are the cause of the observed increase in lipid peroxidation and the differences in their increase between maize and sorghum partly explain the differences in drought sensitivity between these two species.

4.1.6 Drought stress alters the activity of antioxidant enzymes

During ROS-induced oxidative stress, plants induce the activity of antioxidant enzymes to detoxify ROS, preventing their excessive accumulation and contributing to plant survival (Blokhina *et al*., 2003).

In this study, the antioxidant enzymes profiles of SOD, APX, CAT, and GR were evaluated in *Zea mays* and *Sorghum bicolor* to understand how they were influenced by water deprivation.

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes acting as a first-line of plant defence against oxidative stress, converting superoxide to H_2O_2 and O_2 (Mittler, 2002). In this study, under water stress, the overall SOD activity was increased in the leaves of *Zea mays*, while no significant difference was observed in *Sorghum bicolor*. This increase in SOD activity in maize could be as a result of the overproduction of $O_2^{\cdot}$. This is consistent with the study of Luna *et al*. (1985), which found that maize had higher SOD activity than wheat when exposed to water stress. On the other hand, drought inhibited SOD activity in the roots of both maize and sorghum. The inhibition in SOD activity by drought in maize roots could be the reason for the increase in superoxide content. Although the SOD activity in sorghum roots was also decreased under water deficit, no significant changes in superoxide accumulation was observed. These results suggest that *Sorghum bicolor* has more efficient SOD capacity than *Zea mays*, resulting in less accumulation of $O_2^{\cdot}$ in sorghum than in maize.

Under drought conditions, both plant species increased total APX activity in the leaves, which could be attributed to efforts to prevent oxidative damage caused by the increased levels of H_2O_2. Similar results where APX activity increased in response to water stress were reported by Zlatev *et al*. (2006) in bean and Chugh *et al*. (2011) in *Zea mays*. However, APX activity was decreased in the roots of *Zea mays*, while APX activity remained unchanged in the roots of sorghum. The higher increase of APX activity in the leaves of maize is likely a response to the higher H_2O_2 content in maize than in sorghum in response to drought.

Drought stress significantly decreased the activity of CAT in the leaves and roots of both maize and sorghum. A decrease in CAT activity was also observed in a study reported by Pan

et al. (2006) in liquorice and Bakalova *et al*. (2004) in wheat. Reduction of CAT activity is likely the underlying reason for the elevation of H_2O_2 in response to drought.

Glutathione reductase is very important to retain a reduced glutathione pool by reducing glutathione disulphide (GSSG) to the sulfhydryl form (GSH) (GSH is an electron donor for DHAR, which reduces DHA to ascorbate) (Meloni *et al*., 2003). In this study, the activity GR increased in the leaves of *Zea mays*, while there was no statistical difference in *Sorghum bicolor* in response to drought stress. This increase of GR activity in the leaves of maize could be attributed to the significant increase of APX activity in the leaves of maize as the cells may be driving GR activity for regeneration of both GSH and ascorbate. On the other hand, GR activity was decreased in the roots of *Zea mays* while a significant increase was observed in the roots of *Sorghum bicolor*. The decrease in GR activity in the roots of maize might similarly be due to the significant reduction of APX activity in the roots of maize as the cells may have less need for driving GR activity for regeneration of both GSH and ascorbate.

4.2 Drought-responsive leaf proteins of *Zea mays* and *Sorghum bicolor* revealed by proteomics

4.2.1 (SDS / Phenol) Extraction and 1D SDS-PAGE

Protein extraction for this study involved mechanical disruption of the plant tissue using polyvinylpolypyrrolidone (PVPP; Sigma) to remove the polyphenolic compounds (Erhard and Gross, 2006) which interfere with gels and/or LC/MS analysis. This step was done under liquid nitrogen to inhibit proteolysis that may lead protein degradation. Extraction of the proteins with the phenol / SDS combination has resulted in decent quality proteins that do not contain interfering substances, evidenced by no visible signs of streaking and smearing.

4.2.2 Identification of differentially expressed proteins between *Zea mays* and *Sorghum bicolor* in response to drought stress

In this study, 3154 distinguishable proteins were successfully identified by label-free mass spectrometry analysis. This high identification success can be attributed to the use of label-free gel-free LC-MS. Label-free quantitation can more accurately estimate the abundance of proteins than gel-based methods and can detect the differential proteins abundance in a greater

dynamic range than the labelling techniques (Neilson *et al.*, 2011). This is due to the fact that the label-free method employs high-performance liquid chromatography combined with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Nepomuceno *et al*., 2013).

Interestingly, two antioxidant enzymes were detected among the identified proteins which their protein abundance differs between *Zea mays* and *Sorghum bicolor*. These findings could support the results obtained in section 3.1 which revealed that the antioxidant defense system of two plant species respond differently under deprivation.

Of the 207 proteins differentially expressed between maize and sorghum under normal conditions, eight proteins have been identified to be differentially expressed between well-watered and water-deprived plants which appeared to define how the two plant species differently respond to drought stress. Identified proteins showed varying degrees of expression, however, 4 proteins showed an increased abundance in both maize and sorghum and 4 were down-represented in maize and sorghum, with different fold-changes.

4.2.3 Ontological classification of differentially expressed proteins between *Zea mays* and *Sorghum bicolor* in response to water deprivation

Prediction of protein subcellular localization is an important part of identifying their functions and interactions in biological systems (Emanuelsson, 2002).

The identified proteins, were grouped into categories determined according to the annotation presented in OrthoDB (https://www.orthodb.org), UniProtKB (https://www.uniprot.org/help/uniprotk), OMA orthology database (https://omabrowser.org/oma/home/), WoLF PSORT (https://wolfpsort.hgc.jp) and literature sources based on their sub-cellular localization. Most of the characterized proteins were found to be localized in the chloroplast, followed by cytoplasm and cell membrane, with the rest (a much smaller fraction) in other localizations. These findings are consistent with the results observed in sorghum (Kumar *et al.*, 2011) and a study done by Friso *et al*. (2010) on maize. Chloroplasts are very important organelles which perform a wide range of metabolic functions playing a vital role in plant growth and development (Zybailov *et al.*, 2008). Therefore, the results observed in the present study correlate with the dominance of chloroplast as an essential cellular component in photosynthesizing plants.

Moreover, identified proteins were also functionally classified into 9 different categories (Figure3-15). Several proteomics studies showed significant changes in plant metabolism that seem to direct energy towards defense mechanisms in response to water stress to avoid severe damage (Ali and Komatsu, 2006; Xiao *et al.*, 2009; Cramer *et al.*, 2013). In the present work, most of the differentially expressed proteins were related to metabolism and energy, which suggests their importance in drought responses of maize and sorghum. Previous studies indicated that carbohydrate metabolism changes are associated with energy distribution in defence mechanisms of plant to stress (Baena-González, 2010; Morkunas and Ratajczak, 2014). These suggestions are in agreement with results obtained in the present study, where 116 proteins (which represent 56%) of the regulated orthologous protein groups between *Zea mays* and *Sorghum bicolor* are metabolism- and energy-related. Interestingly, among the proteins involved in metabolism, 17 proteins had higher abundance in *Sorghum bicolor* than *Zea mays* in response to water deprivation. Moreover, of the 17 over-represented proteins in sorghum, 4 were not detected in maize (including Frigida-like protein, Tyrosine aminotransferase, Ubiquinol oxidase and Beta-hexosaminidase 1) and 2 showed a decreased abundance in maize. The up-regulation of these proteins in *Sorghum bicolor* suggests their potential role in drought tolerance in *Sorghum bicolor*. Therefore additional investigations are required to determine their activities and interactions in the biological system to further elucidate their role in plant metabolism during exposure to water stress. However, among the eight differentially expressed proteins between well-watered and drought-exposed plants, 3 orthologs are associated with energy, 2 proteins are involved in plant metabolism, 2 proteins are associated to signal transduction mechanisms and 1 protein is associated with protein fate. The potential roles of these proteins are discussed below.

Changes in metabolism-related proteins in response to drought

Proteins involved in metabolism included sucrose synthase, Phenylalanine/tyrosine ammonia-lyase (PTAL) and Ricin-type beta-trefoil lectin domain-like. Sucrose synthase abundance was decreased by approximately1.9-fold in *Zea mays* and 3.9-fold in *Sorghum bicolor*. Sucrose synthase participates in sucrose degradation to form D-fructose and UDP-glucose (or ADP-glucose), which is then used for the biosynthesis of cell walls (Lunn, 2002). Significant

correlation between low sucrose synthase activity and increased sucrose content in a drought-tolerant cultivar of wheat was reported by Kaur (Kaur *et al*., 2007). Sucrose accumulation plays a role in mediating osmotic adjustment to maintain water balance in the cell (Burg and Ferraris, 2008). Interestingly, the decrease in sucrose synthase abundance was more pronounced in sorghum under water deprivation than drought-stressed maize plants. The more pronounced decrease in abundance of sucrose synthase in *Sorghum bicolor* possibly contributes to the ability of sorghum to retain water more efficiently than maize under water deficit. The enzyme phenylalanine/tyrosine ammonia lyase (PTAL) is the key enzyme in the metabolism of polyphenols, which catalyses the non-oxidative deaminating reaction of L-phenylalanine and L-tyrosine to form trans-cinnamic acid and p-coumaric acid. These enzymes facilitate the phenylpropanoid biosynthesis pathway which produces molecules that act as antioxidants. The gene expression of PTAL increased in salted-stressed *Zea mays* (Ertani and Schiavon, 2013). Opposite results were obtained both in this work and in stressed *Medicago sativa L.* (Orr *et al*., 1993), where a decrease in PAL expression was associated with increased accumulation of free trans-cinnamic acid (CA). Assessment of flavonoid contents will thus be an appropriate investigation to better understand the contribution of these enzymes to the phenylpropanoid pathway in response to drought in maize and sorghum. The second protein of interest associated with metabolism is Ricin-type beta-trefoil lectin domain-like, which can be defined as a carbohydrate binding protein. Lectins are involved in plant signaling and/or defense (De Schutter *et al.*, 2017). Ricin-type beta-trefoil lectin domain-like was over-presented in both *Zea mays* (3.4 fold change) and *Sorghum bicolor* (0.4 fold change). The over-representation of this stress responsive protein in *Zea mays* might be related to its sensitivity to water deficit.

Changes in the group of proteins involved in energy metabolism

Two proteins involved in energy metabolism were upregulated in response to water deficit in both maize and sorghum. Indole-3-acetaldehyde oxidase, which is involved in the auxin biosynthesis and in the biosynthesis of abscisic acid (ABA) (Seo *et al*., 1998) was upregulated in both plant species. Even though water-deprived *Zea mays* plants exhibited higher fold

change (5.0) than water-deprived sorghum plants (1.2), the abundance of indole-3-acetaldehyde oxidase was significantly greater in *Sorghum bicolor* under both water conditions when compared to *Zea mays*. In maize, spectral count increased from 1 to 6 in response to drought, while sorghum exhibited 13 spectra under well-watered conditions, and 28 detectable spectra were evident in water-stressed sorghum plants. Auxin is associated with almost all aspects of plant growth including cell elongation, cell division and cell differentiation (Seo *et al*., 1998). As a regulator and coordinator, auxin can influence plant growth and development under stress (Guo *et al.*, 2018). In this study, the higher abundance of indole-3-acetaldehyde oxidase in *Sorghum bicolor* than in maize could be responsible for the less reduction of shoot length and weight in sorghum. Abscisic acid (ABA) is an important plant hormone involved in plant acclimation to various abiotic stresses and in controlling leaf stomatal conductance *(*Min *et al*., 2000). Therefore, the over-representation of indole-3-acetaldehyde oxidase protein in *Sorghum bicolor* related to its better drought tolerance since the enzyme is involved in ABA biosynthesis.

A second protein related to the energy metabolism is citrate synthase, which was more abundant in sorghum then in maize. Maize exhibited a 1.7-fold increase in response to water deficit, rising from 3 to 8 spectral counts, while a more pronounced increase (4.5-fold) was evident in drought-subjected sorghum plants, for which the number of spectra improved from 2 to 11 as a result of plant exposure to water stress. Citrate synthase belongs to a small enzyme family catalyzing the first reaction in the Krebs' cycle (Wu and Minteer, 2015). It converts oxaloacetate and acetyl-coenzyme A into citrate and coenzyme A (Salminen *et al.*, 2014). This is considered to be a crucial reaction for energy production and carbon assimilation. The over-representation of citrate synthase enzyme in this study is consistent with a study done by (Ma *et al.*, 2001) which demonstrated an increase in citrate synthase activity and amount of mRNA in aluminum-tolerant *Paraserianthes falcataria*. Thus, increases in citrate synthase abundance observed in *Sorghum bicolor* would strongly support its better capacity for energy production which plays a key role in plant defense mechanisms to drought stress (Ali and Komatsu, 2006; Xiao *et al.*, 2009; Cramer *et al.*, 2013).

Changes in the signal transduction-related proteins

In the signal transduction category, two different proteins, namely a hypothetical protein (Kinase/pyrophosphorylase) and Tyrosinase/Tyrosine-dopa oxidase were identified. Kinase/pyrophosphorylase, also called pyruvate phosphate dikinase (PPDK) catalyses the reversible reaction that convert ATP and pyruvate to adenine monophosphate (AMP) and phosphoenolpyruvate (PEP). PEP acts as the primary CO_2 acceptor molecule in the C4 photosynthesis pathway in higher plants (Hýsková *et al.*, 2014). (Stenzel, 2010) reported that increased PPDK activity was associated with organic acid exudation and enhanced aluminium stress tolerance in tobacco plants. A study done by Jedmowski *et al.* (2014) showed an increase in PPDK expression in sorghum under drought stress. In Arabidopsis, up-regulation of PPDK upon senescence can enhance the remobilization of nitrogen which increases the growth rate of rosettes as well as the seed weight and nitrogen content (Taylor *et al*., 2010). Opposite results were reported both in the present work and in two *Sorghum bicolor* cultivars with different drought sensitivity characteristics when they were exposed to severe drought conditions (Beyel and Bru, 2005). In these instances, only a limited decrease of PPDK activity was observed in the drought-tolerant cultivar than in the sensitive one. This suggests that restrained decline in PPDK expression in *Sorghum biolor* (0.6–fold) than in *Zea mays* (2-fold change) might be related to sorghum tolerance to water deficit. Thus, further investigation to elucidate the role of PPDK in drought stress might be beneficial towards improvement of plant adaptation to drought stress. Although tyrosinase/Tyrosine-dopa oxidase protein expression was upregulated by 8.0- and 2.6-fold respectively in *Zea mays* and *Sorghum bicolor*, it was significantly more abundant in sorghum under both control and stress conditions. Tyrosinase/Tyrosine-dopa oxidase is involved in the production melanins and other polyphenolic compounds by catalyzing the hydroxylation of monophenols and the oxidation of DOPA to DOPA-quinone (Manga and Orlow, 2011). The role of tyrosinase in plant adaptation to stress has not been clearly defined. However, previous work by Mastore *et al*. (2005) revealed that during melanogenesis, H_2O_2 is involved in the oxidations of DOPA and dopamine. This was supported by a study by Yamazaki *et al*. (2004) which reported that tyrosinase may also exhibit catalase and peroxidase activity. In summary, tyrosinase can directly be involved in plant detoxification of ROS by converting superoxide into O_2 and H_2O,

and/or contribute to plant protection against stress as a key enzyme in the biosynthesis of phenolic compounds which play a significant role in plant defense and response to drought stress (Siger and LAMPART-SZCZAPA, 2008; Lim *et al.*, 2012).

Therefore, the over-representation of tyrosinase in *Sorghum bicolor* under both water regimens could explain the better sorghum adaptation to drought stress.

Changes in the expression of proteins regulating protein fate (folding, modification and destination)

In this group of proteins, phospholipase D (PLD) was upregulated by drought stress in both plant species. The expression change of phospholipase D in *Zea mays* was found to be more (3.2-fold change) than in *Sorghum bicolor* (0.8-fold increase. These results are consistent with Hong *et al.* (2016), who reported the overexpression of PLD genes in Arabidopsis, rice, and tomato when exposed to different pathogens and its activation under heat stress in tobacco (Hou *et al*., 2016). This suggests that phospholipase D (PLD) is triggered in response to various environmental stresses. PLD is a calcium-dependent enzyme which catalyses the hydrolysis of glycerol-phospholipids at the terminal phosphodiester bond, generating phosphatidic acid (PA) and a free head group (Hong *et al.*, 2016). It has reported that PLD is considered to be a key enzyme in plant physiology, required for ABA-mediated stomatal closure, which reduces plant water loss when exposed to water deprivation (Hou *et al*., 2016). The higher expression of PLD observed in maize than sorghum indicates the higher sensitivity of maize to water stress.

Chapter Five

Conclusion and Future Work

Plant responses to drought vary depending on the duration and severity of the stress. Such responses are also determined by the plant species, genotype, age and stage of development (Kim *et al*., 2012). As the molecular basis of plant drought tolerance is still not fully understood, reduced crop production is endangering global food security (Heinemann *et al*., 2017). Thus, it is essential to improve crop production stability under water limited environments in order to sustain the food security for the growing world population (Basu *et al.*, 2016).

We conducted comparative analyses of the responses of *Zea mays* and *Sorghum bicolor* to drought stress through incorporating some of the important physiological and biochemical measurements and changes in the proteomic profiles in an attempt to understand the mechanisms underlying better adaptation of sorghum to drought stress than maize. Investigation of the response of enzymatic antioxidant activities between maize and sorghum exhibited mostly similar trends but with a few differences in specific isoforms of each of the antioxidant enzymes. Furthermore, a decrease in chlorophyll content was also evident in both plant species. Drought stress significantly inhibited the growth of *Zea mays*, decreased the RWC, and increased ROS, MDA content and cell damage. In contrast, the impact of drought stress in *Sorghum bicolor* was less pronounced as less effect on growth and the level oxidative stress was observed compared to *Zea mays*. In addition, sorghum significantly induced the accumulation of free proline in the roots and, at the same time, displayed better ability to maintain good water status. In conclusion, the physiological and biochemical results in this work support the hypothesis that *Sorghum bicolor* is more drought tolerant than *Zea mays*. Similar results in two contrasting cultivars of wheat were reported by Herbinger *et al*. (2002). Importantly, the proteomic profiling of the leaves of these two cereal crops revealed obvious differences in the protein patterns. Differentially regulated proteins between maize and sorghum reported in this work are mostly involved in plant metabolism and energy, protein

fate and signal transduction; suggesting that changes in proteins related to these functional categories play an important role in plant acclimation to drought stress. These results suggest that better acclimation of *Sorghum bicolor* than maize to water deficit involves changes in some of the drought-responsive proteins such as sucrose synthase enzyme which involved in sucrose degradation. Given that sucrose is one of the important osmolytes that regulate the osmotic adjustment in plants, it is then understandable why sorghum is better at maintaining a good water status than maize. In summary, the significant decreased abundance of sucrose synthase in *Sorghum bicolor* under water deprivation could contribute to its better capability to retain water than maize. The differential regulation of energy-related proteins such as Indole-3-acetaldehyde oxidase could be related to the better growth of *Sorghum bicolor* under drought stress. Changes in tyrosinase / tyrosine-dopa oxidase might also be related to the efficient capacity of sorghum of controlling ROS accumulation since tyrosinase / Tyrosine-dopa oxidase has catalase and peroxidase activity (Yamazaki *et al*., 2004). Work is in progress to integrate the proteomics studies presented here with on-going transcriptomics studies in these two cereal species. Moreover, since the root plays a major role in plant osmotic responses and little is known about proteomic changes in maize and sorghum from a comparative proteomics point of view (Kiegle *et al*., 2000), it would be interesting in the future to study the root profiles of the two cereal species.

Chapter Six

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Appendix

Table 3-6 Differentially expressed proteins between *Z. mays* and *S. bicolor* in response to drought as identified by LC-MS

Accession No	ID	Cellular component	Q-value (Species)	Q-value (Treatment)	Spectral count				Fold change	
					Z. mays WW	*Z. mays* WD	*S. bicolor* WW	*S. bicolor* WD	*Z. mays*	*S. bicolor*
Metabolism										
GRMZM2G152908_P01 Sobic.001G344500.2.p	Sucrose synthase (E2.4.1.13)	Cytoplasm	1.5E-06	0.0001	106	37	54	11	-1.9	-3.9
GRMZM2G074604_P01 Sobic.004G220300.1.p	Phenylalanine/tyrosine ammonia-lyase (PTAL)	Cytoplasm	2.1E-06	0.0036	97	51	51	25	-0.9	-1.0
GRMZM2G120304_P02 Sobic.003G105700.1.p	Ricin-type beta-trefoil lectin domain-like	Cytoplasm	7.0E-06	0.0142	5	22	8	11	3.4	0.4
GRMZM2G339563_P01 Sobic.001G504900.1.p	Pheophorbide a oxygenase	Chloroplast	0.0002	0.1159	6	13	5	17	1.2	2.4
GRMZM2G175718_P01 Sobic.002G361400.1.p	Frigida-like protein	Nucleus	0.0003	0.1159	0	0	3	8	0.0	1.7
GRMZM2G412604_P01 Sobic.002G041100.1.p	Tyrosine aminotransferase	Membrane	0.0006	0.1274	0	0	4	13	0.0	2.3

GRMZM2G089713_P07 Sobic.010G072300.1.p	Glycosyltransferase	Cytoplasm	0.0008	0.1490	45	32	38	20	-0.4	-0.9
GRMZM2G413647_P01 Sobic.009G233900.1.p	2-hydroxy-3-oxopropionate reductase	Integral component of membrane	0.0009	0.1880	8	3	5	2	-1.7	-1.5
GRMZM2G035256_P01 Sobic.003G029200.1.p	Lecithin-cholesterol acyltransferase-related	Membrane	0.0010	0.1950	8	9	14	23	0.1	0.6
GRMZM2G144668_P01 Sobic.003G039700.1.p	Oxidoreductase, 2og-fe ii oxygenase family protein	Integral component of membrane	0.0010	0.2232	8	10	23	38	0.3	0.7
GRMZM2G052812_P02 Sobic.010G019800.1.p	Aldo-keto reductase 1-related	Cytoplasm	0.0011	0.2232	14	16	22	37	0.1	0.7
GRMZM2G050072_P01 Sobic.003G219700.1.p	10-deacetylbaccatin III 10-O-acetyltransferase	Chloroplast	0.0012	0.2316	9	19	3	14	1.1	3.7
GRMZM2G178958_P01 Sobic.004G329300.1.p	Leucyl aminopeptidase	Intracellular	0.0019	0.2586	97	106	83	141	0.1	0.7
GRMZM2G456086_P01 Sobic.002G051100.1.p	Ribose-5-phosphate isomerase	Cytoplasm	0.0021	0.2592	23	17	33	21	-0.4	-0.6
GRMZM2G032003_P01 Sobic.002G291200.1.p	UTP--glucose-1-phosphate uridylyltransferase	Cytosol	0.0021	0.2592	82	109	99	110	0.3	0.1
GRMZM2G110567_P01 Sobic.006G009000.1.p	Calcium-activated chloride channel regulator	Endoplasmic reticulum	0.0022	0.2592	3	23	10	12	6.7	0.2
GRMZM2G054465_P01 Sobic.010G198000.1.p	Tryptophan synthase beta chain	Chloroplast	0.0025	0.2854	0	3	7	13	3.0	0.9

GRMZM2G088396_P01 Sobic.004G053700.1.p	4-hydroxyphenylpyruvate dioxygenase	Cytoplasm	0.0026	0.2854	8	16	2	5	1.0	1.5
GRMZM2G075333_P01 Sobic.004G062500.1.p	4-coumarate--coA ligase	Integral component of membrane	0.0026	0.2854	17	5	1	1	-2.4	0.0
GRMZM2G419806_P03 Sobic.008G051000.1.p	Magnesium chelatase subunit I	Chloroplast	0.0026	0.2854	57	30	27	23	-0.9	-0.2
GRMZM2G074331_P01 Sobic.009G205700.1.p	Glucosyl/glucuronosyl transferases	Membrane	0.0030	0.2908	4	1	12	32	-3.0	1.7
AC196475.3_FGP004 Sobic.007G047300.1.p	Caffeic acid 3-O-methyltransferase	Cytosol	0.0031	0.2908	60	31	18	18	-0.9	0.0
GRMZM2G098290_P02 Sobic.006G249400.1.p	Glutamine synthetase, chloroplastic/mitochondrial	Nucleus	0.0031	0.2908	45	26	19	16	-0.7	-0.2
GRMZM2G060659_P02 Sobic.010G276700.1.p	Glycosyltransferase	Integral component of membrane	0.0032	0.2908	59	31	22	21	-0.9	0.0
GRMZM2G170812_P01 Sobic.001G368600.1.p	Nad(p)-binding rossmann-fold superfamily protein	Chloroplast	0.0034	0.2908	28	19	15	4	-0.5	-2.8
GRMZM2G172369_P03 Sobic.005G132400.1.p	Alpha-mannosidase	Cell wall	0.0045	0.2908	20	14	25	16	-0.4	-0.6
GRMZM2G035213_P01 Sobic.004G269800.1.p	Tocopherol O-methyltransferase	Chloroplast	0.0047	0.2908	4	5	0	8	0.3	8.0
GRMZM2G088064_P03 Sobic.001G260800.1.p	Alanine transaminase	Cytoplasm	0.0047	0.2908	10	18	0	0	0.8	0.0

GRMZM2G136453_P01 Sobic.007G194100.1.p	Acid phosphatase related	Membrane	0.0047	0.2948	0	15	5	3	15.0	-0.7
GRMZM2G113332_P01 Sobic.004G164200.1.p	Copper transport protein	Chloroplast	0.0051	0.3066	29	27	31	14	-0.1	-1.2
GRMZM2G101142_P01 Sobic.002G373100.1.p	Strictosidine synthase-related	Integral component of membrane	0.0051	0.3066	2	11	8	11	4.5	0.4
GRMZM5G815453_P01 Sobic.005G194400.1.p	Ribulose-bisphosphate carboxylase large chain	Chloroplast	0.0051	0.3075	394	309	148	126	-0.3	-0.2
GRMZM2G074097_P01 Sobic.002G384400.1.p	Thiamine thiazole synthase	Cytosol	0.0054	0.3075	52	28	12	15	-0.9	0.3
GRMZM2G044237_P03 Sobic.001G416700.1.p	Glutamate N-acetyltransferase	Chloroplast	0.0071	0.3089	5	8	9	13	0.6	0.4
GRMZM2G104310_P03 Sobic.008G039900.1.p	Glycine dehydrogenase	Cytoplasm	0.0072	0.3089	38	20	49	41	-0.9	-0.2
GRMZM2G328094_P01 Sobic.003G110200.1.p	Alcohol dehydrogenase related	Cytoplasm	0.0080	0.3089	19	0	6	2	-19.0	-2.0
GRMZM2G126010_P03 Sobic.009G153000.1.p	Actin	Cytoplasm	0.0080	0.3089	13	15	6	17	0.2	1.8
GRMZM2G155253_P02 Sobic.005G056400.1.p	Fructose-bisphosphate aldolase 2, chloroplastic-related	Chloroplast	0.0089	0.3089	143	92	123	116	-0.6	-0.1
GRMZM2G126002_P01 Sobic.008G097000.1.p	L-galactose dehydrogenase	Cytosol	0.0090	0.3089	0	0	11	4	0.0	-1.8

GRMZM2G050514_P03 Sobic.001G451500.1.p	Glutamine synthetase	Cytoplasm	0.0102	0.3132	1	9	7	8	8.0	0.1
GRMZM2G177923_P01 Sobic.006G157700.1.p	Beta-d-xylosidase 6-related	Extracellular region	0.0109	0.3323	8	5	3	0	-0.6	-3.0
GRMZM2G306732_P01 Sobic.001G425400.1.p	Fructose-1,6-bisphosphatase	Chloroplast	0.0109	0.3663	94	73	81	70	-0.3	-0.2
GRMZM5G852968_P03 Sobic.002G277100.1.p	Triosephosphate isomerase, chloroplastic	Cytosol	0.0109	0.3765	97	74	107	94	-0.3	-0.1
GRMZM2G165747_P01 Sobic.001G148900.2.p	Cobalamin-independent methionine synthase	Cytoplasm	0.0109	0.3794	54	33	58	54	-0.6	-0.1
GRMZM2G057000_P03 Sobic.010G277300.1.p	Delta24-sterol reductase	Endoplasmic reticulum	0.0112	0.3940	9	4	4	3	-1.3	-0.3
GRMZM2G155962_P03 Sobic.006G054300.1.p	4-sulfomuconolactone hydrolase	Cytoplasm	0.0145	0.4061	5	6	15	23	0.2	0.5
GRMZM2G126261_P01 Sobic.002G416700.1.p	Peroxidase / lactoperoxidase	Extracellular region	0.0147	0.4157	14	18	1	5	0.3	4.0
GRMZM5G845611_P01 Sobic.001G519800.1.p	Glyceraldehyde-3-phosphate dehydrogenase	Chloroplast	0.0172	0.4283	227	193	199	189	-0.2	-0.1
GRMZM2G169516_P02 Sobic.006G112600.1.p	Indole-3-glycerol phosphate synthase, chloroplastic	Chloroplast	0.0174	0.4283	9	3	6	3	-2.0	-1.0
GRMZM5G844195_P02 Sobic.001G052700.1.p	Vacuolar protein sorting-associated protein 35	Golgi apparatus	0.0181	0.4294	6	1	8	7	-5.0	-0.1

GRMZM2G101875_P02 Sobic.009G031400.1.p	Long chain acyl-coA synthetase 1	Membrane	0.0188	0.4399	18	10	2	1	-0.8	-1.0
GRMZM2G136106_P01 Sobic.003G148400.1.p	Pectinesterase inhibitor 39-related	Cell wall	0.0188	0.4399	6	4	15	10	-0.5	-0.5
GRMZM2G323024_P01 Sobic.006G264900.1.p	Magnesium chelatase subunit H (chlh, bchh)	Chloroplast	0.0203	0.4399	10	1	9	4	-9.0	-1.3
GRMZM2G442804_P03 Sobic.010G207100.1.p	Demethylmenaquinone methyltransferase	Chloroplast	0.0207	0.4401	0	2	7	12	2.0	0.7
GRMZM2G123204_P01 Sobic.001G126300.1.p	Adenylosuccinate synthase	Cytoplasm	0.0231	0.4504	20	12	15	12	-0.7	-0.3
GRMZM2G081462_P01 Sobic.003G132100.1.p	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic	Chloroplast	0.0240	0.4559	34	23	24	20	-0.5	-0.2
GRMZM2G013478_P01 Sobic.008G123600.1.p	Nucleoside diphosphate kinase 2	Chloroplast	0.0240	0.4559	23	20	27	21	-0.2	-0.3
AC233960.1_FGP002 Sobic.004G270500.1.p	Ubiquinol oxidase	Integral component of membrane	0.0246	0.4559	0	0	4	8	0.0	1.0
GRMZM2G009196_P01 Sobic.008G118600.1.p	Riboflavin synthase	Chloroplast	0.0262	0.4616	16	17	16	9	0.1	-0.8
GRMZM2G159016_P01 Sobic.009G247100.1.p	Vitellogenic carboxypeptidase-like protein	Chloroplast	0.0265	0.4643	4	6	49	30	0.5	-0.6

GRMZM2G179981_P01 Sobic.004G130800.2.p	Cinnamyl-alcohol dehydrogenase-related	Cytosol	0.0288	0.4643	11	17	5	5	0.5	0.0
GRMZM2G074282_P02 Sobic.004G001300.1.p	D-isomer specific 2-hydroxyacid dehydrogenase, NAD binding domain	Chloroplast	0.0324	0.4643	38	25	52	47	-0.5	-0.1
GRMZM2G391936_P05 Sobic.001G100000.1.p	Glucose-1-phosphate adenylyltransferase large subunit 1, chloroplastic	Amyloplast	0.0331	0.4643	65	49	93	79	-0.3	-0.2
GRMZM2G107639_P02 Sobic.003G045600.1.p	Subgroup i aminotransferase related	Cytoplasm	0.0332	0.4643	1	1	5	13	0.0	1.6
GRMZM2G426415_P01 Sobic.005G172900.1.p	Hydroquinone glucosyltransferase	Chloroplast	0.0355	0.4643	3	9	4	4	2.0	0.0
GRMZM2G393671_P01 Sobic.002G371400.2.p	Stress responsive A/B Barrel Domain (Dabb)	Integral component of membrane	0.0355	0.4643	11	8	10	5	-0.4	-1.0
GRMZM2G152827_P01 Sobic.004G310300.1.p	Solute carrier family 25 (mitochondrial phosphate transporter), member 3	Integral component of membrane	0.0356	0.4643	25	19	25	17	-0.3	-0.5
GRMZM2G034598_P01 Sobic.009G017500.1.p	Beta-hexosaminidase 1	Extracellular region	0.0356	0.4643	0	0	5	8	0.0	0.6
GRMZM2G128219_P01 Sobic.004G186500.2.p	Member of 'gdxg' family of lipolytic enzymes	Cytoplasm	0.0370	0.4655	0	1	4	8	1.0	1.0

GRMZM2G012863_P01 Sobic.010G073500.1.p	Eta-ketoacyl-[acyl-carrier-protein] synthase I / KAS I	Chloroplast	0.0374	0.4665	8	6	5	13	-0.3	1.6
GRMZM2G477236_P01 Sobic.004G024600.1.p	Beta-fructofuranosidase / Saccharase	Membrane	0.0379	0.4708	10	8	7	1	-0.3	-6.0
GRMZM2G035268_P03 Sobic.007G140700.1.p	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Integral component of membrane	0.0396	0.4708	19	25	23	29	0.3	0.3
GRMZM2G105539_P01 Sobic.004G238200.1.p	Arsenite-transporting atpase	Cytoplasm	0.0428	0.4733	5	3	4	1	-0.7	-3.0
GRMZM2G162529_P01 Sobic.004G272100.1.p	Phosphoribulokinase / phosphopentokinase	Cytosol	0.0428	0.4775	104	69	154	150	-0.5	0.0
GRMZM2G339699_P01 Sobic.003G372400.2.p	Phosphoribosyldiphosphate 5-amidotransferase	Chloroplast	0.0438	0.4775	6	2	7	4	-2.0	-0.8
GRMZM2G035620_P01 Sobic.003G179000.1.p	Carboxymethylenebutenolidase	Chloroplast	0.0109	0.3819	7	17	12	15	1.4	0.3
GRMZM2G098423_P01 Sobic.010G214100.1.p	D-3-phosphoglycerate dehydrogenase	Chloroplast	0.0443	0.4775	7	6	11	5	-0.2	-1.2
GRMZM2G314898_P01 Sobic.003G037800.1.p	Transferase family	Cytoplasm	0.0450	0.4778	25	16	21	22	-0.6	0.0

Energy										
GRMZM2G141473_P01 Sobic.001G062300.1.p	Indole-3-acetaldehyde oxidase	Cytoplasm	1.6E-05	0.0444	1	6	13	28	5.0	1.2
GRMZM2G135588_P01 Sobic.004G101900.1.p	Citrate synthase, peroxisomal	Peroxisome	2.6E-05	0.0458	3	8	2	11	1.7	4.5
GRMZM2G021256_P01 Sobic.004G191200.1.p	PSBQ-LIKE PROTEIN 1, CHLOROPLASTIC (Oxygen-evolving enhancer protein 3)	Chloroplast	2.6E-05	0.0591	15	5	26	15	-2.0	-0.7
GRMZM2G021846_P01 Sobic.009G056700.1.p	6-phosphofructo-2-kinase	Cytoplasm	0.0002	0.0602	10	2	12	3	-4.0	-3.0
GRMZM5G800980_P01 Sobic.003G172966.1.p	NAD(P)H-quinone oxidoreductase subunit K, chloroplastic	Chloroplast	0.0005	0.1274	13	5	6	3	-1.6	-1.0
GRMZM2G181505_P01 Sobic.004G246200.1.p	Dihydropyrimidine dehydrogenase	Cytoplasm	0.0005	0.1274	7	19	11	17	1.7	0.5
GRMZM2G139512_P02 Sobic.006G041000.1.p	Alcohol dehydrogenase	Cytoplasm	0.0009	0.1917	8	23	12	16	1.9	0.3
GRMZM2G123029_P05 Sobic.009G034700.5.p	Phytepsin	Vacuole	0.0010	0.2232	0	1	15	27	1.0	0.8
GRMZM2G025171_P02 Sobic.004G235200.1.p	F-type H+-transporting atpase subunit delta	Membrane	0.0011	0.2232	43	26	59	45	-0.7	-0.3

GRMZM2G085019_P01 Sobic.003G036200.1.p	Malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+)	Chloroplast	0.0011	0.2232	188	128	128	92	-0.5	-0.4
GRMZM2G039396_P01 Sobic.003G137100.1.p	Oxygen-dependent protoporphyrinogen oxidase	Integral component of membrane	0.0012	0.2232	17	0	6	4	-17.0	-0.5
GRMZM2G077214_P01 Sobic.001G167900.1.p	Aryl-alcohol dehydrogenase	Chloroplast	0.0026	0.2854	44	31	29	19	-0.4	-0.5
GRMZM2G467338_P02 Sobic.006G000100.1.p	Aconitate hydratase	Cytoplasm	0.0027	0.2854	62	99	55	68	0.6	0.2
GRMZM2G119499_P01 Sobic.001G514400.1.p	Glutathione s-transferase, gst, superfamily	Cytoplasm	0.0034	0.2908	30	23	34	28	-0.3	-0.2
GRMZM2G351977_P02 Sobic.003G209800.1.p	Light harvesting chlorophyll a/b binding protein1	Chloroplast	0.0042	0.2908	33	15	42	27	-1.2	-0.6
GRMZM2G080107_P01 Sobic.008G063500.1.p	Photosystem I subunit psan	Photosystem I	0.0065	0.3089	22	22	58	34	0.0	-0.7
GRMZM2G033894_P01 Sobic.006G011200.1.p	Pyruvate dehydrogenase e1 component subunit alpha-3, chloroplastic	Chloroplast	0.0079	0.3089	9	3	10	8	-2.0	-0.3
GRMZM5G856653_P01 Sobic.003G291800.1.p	Hexokinase	Mitochondria	0.0079	0.3089	18	22	21	26	0.2	0.2
GRMZM2G092311_P02 Sobic.004G056900.1.p	Chlorophyll A-B binding protein	Chloroplast	0.0080	0.3089	33	28	32	18	-0.2	-0.8

GRMZM2G359038_P02 Sobic.004G087500.1.p	ATP:ADP antiporter	Integral component of membrane	0.0099	0.3114	4	11	2	3	1.8	0.5
GRMZM2G109244_P01 Sobic.006G162200.1.p	Cyanobacterial and plastid NDH-1 subunit M (ndhm)	Thylakoid membrane	0.0109	0.3765	31	20	25	22	-0.6	-0.1
GRMZM2G024099_P01 Sobic.001G478100.1.p	Aspartyl protease-like protein	Membrane	0.0141	0.4029	19	3	25	25	-5.3	0.0
GRMZM2G379002_P01 Sobic.004G343500.1.p	Peptidase_S41	Chloroplast	0.0147	0.4061	3	6	0	5	1.0	5.0
GRMZM2G110277_P01 Sobic.003G378600.1.p	Nadh dehydrogenase-like complex n	Chloroplast	0.0149	0.4157	37	37	37	11	0.0	-2.4
GRMZM2G036609_P02 Sobic.002G402700.1.p	Ferredoxin-dependent glutamate synthase	Chloroplast	0.0220	0.4502	75	62	61	41	-0.2	-0.5
GRMZM2G329047_P01 Sobic.002G242000.1.p	Photosystem I subunit V	Membrane	0.0253	0.4605	12	5	10	8	-1.4	-0.3
GRMZM2G105644_P01 Sobic.004G238500.1.p	Geranylgeranyl hydrogenase1	Chloroplast	0.0265	0.4626	47	39	51	47	-0.2	-0.1
GRMZM5G825759_P01 Sobic.001G417200.1.p	F-type H+-transporting atpase subunit b	Mitochondria	0.0304	0.4643	14	15	36	22	0.1	-0.6
GRMZM2G156068_P01 Sobic.004G264700.1.p	F-type H+-transporting atpase subunit O	Membrane	0.0342	0.4643	18	25	30	44	0.4	0.5
GRMZM2G145854_P01 Sobic.001G115700.1.p	NADH dehydrogenase (ubiquinone) Fe-S protein 1	Membrane	0.0370	0.4658	16	15	13	29	-0.1	1.2

GRMZM5G824600_P03 Sobic.004G343200.1.p	Uncharacterized protein (Zinc-binding dehydrogenase)	Extracellular region	0.0376	0.4665	6	6	3	8	0.0	1.7
GRMZM2G432801_P01 Sobic.009G203500.1.p	Hexokinase	Mitochondria	0.0399	0.4733	0	9	24	28	9.0	0.2
GRMZM2G006672_P02 Sobic.004G202100.1.p	Isocitrate dehydrogenase (NAD+)	Mitochondria	0.0404	0.4733	9	14	13	17	0.6	0.3
GRMZM2G021635_P04 Sobic.006G237200.1.p	V-type H+-transporting atpase subunit D	Chloroplast	0.0429	0.4775	17	23	20	25	0.4	0.3
Signal transduction mechanism										
GRMZM2G004880_P01 Sobic.002G324500.1.p	Hypothetical protein (Kinase/pyrophosphorylase)	Chloroplast	3.0E-06	0.0060	29	10	28	17	-1.9	-0.6
GRMZM2G319062_P01 Sobic.007G068500.1.p	Tyrosinase / Tyrosine-dopa oxidase	Chloroplast	5.4E-06	0.0097	0	8	11	40	8.0	2.6
GRMZM2G154090_P01 Sobic.001G234800.1.p	Inorganic phosphate transporter 1-4	Membrane	0.0002	0.1130	0	0	2	9	0.0	3.5
GRMZM5G870446_P02 Sobic.006G102000.1.p	Plastid-lipid-associated protein 3, chloroplastic-related	Chloroplast	0.0004	0.1274	24	29	33	47	0.2	0.4
GRMZM2G300801_P01 Sobic.001G498500.1.p	Phosphoserine transaminase	Cytoplasm	0.0010	0.1958	13	21	7	21	0.6	2.0
GRMZM2G171688_P01 Sobic.006G058800.1.p	Glycyl-trna synthetase/dna polymerase subunit gamma-2	Cytoplasm	0.0012	0.2232	29	19	23	12	-0.5	-0.9

GRMZM2G391364_P01 Sobic.007G019501.1.p	Calcium-binding protein cml14-related	Cytosol	0.0012	0.2232	23	15	23	15	-0.5	-0.5
GRMZM2G010349_P02 Sobic.009G219100.1.p	Serine/threonine-protein kinase stn7, chloroplastic	Integral component of membrane	0.0020	0.2592	28	23	47	36	-0.2	-0.3
GRMZM2G025992_P01 Sobic.002G407900.1.p	Superoxide dismutase, Cu-Zn	Cytoplasm	0.0021	0.2592	0	4	6	9	4.0	0.5
GRMZM2G127141_P02 Sobic.001G315700.1.p	Mitogen-activated protein kinase 11-related	Chloroplast	0.0024	0.2740	5	8	5	14	0.6	1.8
GRMZM2G002178_P02 Sobic.001G449700.1.p	Allene oxide synthase, chloroplastic	Chloroplast	0.0051	0.3066	25	55	29	34	1.2	0.2
GRMZM2G047855_P01 Sobic.001G080700.1.p	Casein kinase II subunit alpha	Nucleus	0.0064	0.3075	0	0	4	10	0.0	1.5
GRMZM2G142413_P01 Sobic.003G042100.3.p	Myosin	Myosin complex	0.0086	0.3089	25	13	19	16	-0.9	-0.2
GRMZM2G436986_P01 Sobic.002G004000.2.p	PSBQ-LIKE PROTEIN 2 (oxygen evolving enhancer protein 3)	Chloroplast	0.0090	0.3089	25	13	26	13	-0.9	-1.0
GRMZM2G032351_P01 Sobic.007G105500.1.p	Phototropin-2	Membrane	0.0130	0.3983	4	10	6	8	1.5	0.3
GRMZM2G022258_P02 Sobic.001G004400.1.p	Exportin-1	Nucleus	0.0174	0.4288	9	7	12	6	-0.3	-1.0

GRMZM2G087590_P01 Sobic.008G065000.1.p	Psbp domain-containing protein 4, chloroplastic	Chloroplast	0.0183	0.4294	12	16	9	12	0.3	0.3
GRMZM2G054354_P01 Sobic.004G272800.5.p	Vhs domain containing protein family	Intracellular	0.0187	0.4294	4	5	3	9	0.3	2.0
GRMZM2G178192_P03 Sobic.007G009200.1.p	Adenylate kinase family protein	Chloroplast	0.0356	0.4643	62	57	69	46	-0.1	-0.5
GRMZM2G169694_P01 Sobic.002G309500.1.p	Ras-related protein Rab-6A	Cytosol	0.0231	0.4504	23	25	24	35	0.1	0.5
GRMZM2G701221_P01 Sobic.009G188300.1.p	Universal stress protein family	Chloroplast	0.0231	0.4504	0	1	4	8	1.0	1.0
GRMZM2G312910_P02 Sobic.002G047400.1.p	Putative tyrosine phosphatase	Chloroplast	0.0240	0.4559	52	58	43	55	0.1	0.3
Protein fate (folding, modification, destination)										
GRMZM2G061969_P01 Sobic.003G050400.1.p	Phospholipase D	Membrane	8.2E-06	0.0155	13	54	25	45	3.2	0.8
GRMZM2G153815_P01 Sobic.001G193500.1.p	Molecular chaperone dnak (heat shock protein)	Extracellular region	0.0002	0.0704	18	25	28	49	0.4	0.8
GRMZM2G162200_P02 Sobic.005G231500.1.p	26S proteasome regulatory complex, atpase RPT4	Cytoplasm	0.0002	0.1159	152	90	71	50	-0.7	-0.4
GRMZM2G028156_P01 Sobic.003G123000.1.p	Splicing factor 3A subunit 1	Nucleus	0.0006	0.1356	0	0	2	9	0.0	3.5

GRMZM2G048324_P01 Sobic.001G342600.1.p	Nucleoredoxin 1-related	Vacuole	0.0030	0.2908	5	12	24	35	1.4	0.5
GRMZM2G027378_P01 Sobic.004G130100.1.p	Ubiquitin-conjugating enzyme e2	Cytoplasm	0.0046	0.2908	28	11	27	19	-1.5	-0.4
GRMZM2G102471_P03 Sobic.003G337000.1.p	Ubiquitin-conjugating enzyme E2 D/E	Nucleus	0.0079	0.3089	4	6	5	9	0.5	0.8
GRMZM2G066996_P01 Sobic.001G168600.1.p	Serpin B (SERINE PROTEASE INHIBITOR)	Extracellular region	0.0080	0.3089	17	21	9	20	0.2	1.2
GRMZM2G084881_P01 Sobic.002G133800.1.p	Disulfide oxidoreductase	Cytoplasm	0.0083	0.3089	28	39	27	34	0.4	0.3
GRMZM2G123922_P01 Sobic.006G058100.1.p	ATP-dependent Clp protease ATP-binding subunit clpc	Cytoplasm	0.0109	0.3605	146	103	121	118	-0.4	0.0
GRMZM2G048836_P01 Sobic.010G092900.1.p	Atp-dependent zinc metalloprotease ftsh 6, chloroplastic	Membrane	0.0109	0.3765	14	13	11	18	-0.1	0.6
GRMZM2G129238_P01 Sobic.002G430500.1.p	Phosphoinositide-specific phospholipase c family protein	Intracellular	0.0109	0.3765	10	14	8	14	0.4	0.8
GRMZM2G015967_P01 Sobic.007G116950.1.p	Peptidyl-prolyl cis-trans isomerase cyp38, chloroplastic	Chloroplast	0.0145	0.4029	79	100	88	106	0.3	0.2
GRMZM2G055684_P01 Sobic.009G151000.1.p	Cucumisin	Chloroplast	0.0145	0.4061	21	7	32	29	-2.0	-0.1

GRMZM2G481843_P01 Sobic.003G071600.1.p	Very-long-chain enoyl-coa reductase	Integral component of membrane	0.0216	0.4401	6	0	3	4	-6.0	0.3
GRMZM2G067242_P01 Sobic.004G272400.1.p	Atpase family aaa domain-containing protein 3	Mitochondria	0.0249	0.4581	4	1	4	2	-3.0	-1.0
GRMZM2G014805_P01 Sobic.008G098900.1.p	Ubiquitin carboxyl-terminal hydrolase 7	Membrane	0.0339	0.4643	7	9	0	5	0.3	5.0
GRMZM2G166646_P01 Sobic.003G034900.2.p	Metalloprotease	Mitochondria	0.0347	0.4643	6	11	10	13	0.8	0.3
GRMZM2G101271_P01 Sobic.004G307100.1.p	Uncharacterized protein (Peptidase M16C associated)	Cytoplasm	0.0356	0.4643	34	34	15	41	0.0	1.7
GRMZM2G068316_P01 Sobic.008G102000.1.p	Dnaj domain	Membrane	0.0356	0.4643	10	12	18	1	0.2	-17.0
GRMZM2G056870_P01 Sobic.005G188100.1.p	20S proteasome subunit alpha 5	Proteasome core complex	0.0399	0.4733	13	16	24	32	0.2	0.3
GRMZM2G134582_P01 Sobic.004G094200.1.p	Elongation factor 1-gamma	Cytoplasm	0.0485	0.4816	25	30	38	55	0.2	0.4
GRMZM2G035708_P04 Sobic.004G234200.3.p	Peptidyl-prolyl cis-trans isomerase fkbp16-2, chloroplastic	Chloroplast	0.0113	0.3940	46	39	39	28	-0.2	-0.4
GRMZM2G009443_P01 Sobic.008G081900.1.p	ATP-dependent Clp protease ATP-binding subunit clpc	Chloroplast	0.0471	0.4811	185	139	162	159	-0.3	0.0

Protein synthesis										
GRMZM2G011129_P01 Sobic.002G131700.1.p	Splicing factor 3b, subunit 4	Nucleolus	0.0012	0.2272	20	8	2	0	-1.5	-2.0
GRMZM2G109677_P02 Sobic.006G216600.1.p	Large subunit ribosomal protein	Ribosome	0.0027	0.2854	38	23	34	25	-0.7	-0.4
GRMZM2G125271_P01 Sobic.001G018900.1.p	40S ribosomal protein S4 C-terminus	Intracellular	0.0072	0.3089	18	10	23	19	-0.8	-0.2
GRMZM2G153476_P01 Sobic.001G122400.1.p	Small subunit ribosomal protein S13	Intracellular	0.0072	0.3089	8	5	13	7	-0.6	-0.9
GRMZM2G156673_P01 Sobic.003G109600.2.p	Ribosomal protein s7p/s5e	Ribosome	0.0083	0.3089	24	26	34	20	0.1	-0.7
GRMZM2G148709_P01 Sobic.009G056200.1.p	Arginyl-trna synthetase (RARS, args)	Cytoplasm	0.0091	0.3089	8	3	7	4	-1.7	-0.8
GRMZM5G851815_P02 Sobic.007G130400.1.p	Lycopene beta-cyclase	Chromoplast	0.0143	0.4029	0	1	3	7	1.0	1.3
GRMZM2G055165_P01 Sobic.006G280050.1.p	30s ribosomal protein s17, chloroplastic	Chloroplast	0.0189	0.4399	5	5	12	6	0.0	-1.0
GRMZM2G041238_P01 Sobic.003G078200.1.p	Large subunit ribosomal protein l26e	Ribosome	0.0210	0.4401	6	10	5	6	0.7	0.2
GRMZM2G138258_P01 Sobic.003G431900.1.p	Nonsense-mediated mRNA decay protein 3	Nucleus	0.0231	0.4504	122	91	61	59	-0.3	0.0

GRMZM2G455085_P01 Sobic.002G304100.1.p	Asparagine--tRNA ligase, mitochondrial	Mitochondria	0.0264	0.4616	7	1	2	2	-6.0	0.0
GRMZM2G113720_P01 Sobic.009G233400.1.p	Large subunit ribosomal protein L18Ae	Ribosome	0.0304	0.4643	5	5	26	14	0.0	-0.9
GRMZM2G018228_P03 Sobic.003G012700.1.p	Small subunit ribosomal protein S15Ae	Cytoplasm	0.0346	0.4643	11	9	20	11	-0.2	-0.8
GRMZM2G152552_P01 Sobic.007G138700.1.p	Large subunit ribosomal protein l34e	Intracellular	0.0355	0.4643	2	4	9	1	1.0	-8.0
GRMZM2G145496_P01 Sobic.003G408000.2.p	Large subunit ribosomal protein L27	Intracellular	0.0356	0.4643	10	9	15	6	-0.1	-1.5
GRMZM2G165137_P01 Sobic.007G091600.1.p	RNA binding protein (contains RRM repeats)	Cytoplasm	0.0369	0.4643	0	0	7	4	0.0	-0.8
GRMZM2G162253_P01 Sobic.002G034500.1.p	Calcium-dependent channel, 7TM region, putative phosphate (RSN1_7TM) // Cytosolic domain of 10TM putative phosphate transporter	Membrane	0.0399	0.4708	6	7	3	8	0.2	1.7
GRMZM2G047727_P01 Sobic.001G444800.1.p	Large subunit ribosomal protein l40e	Ribosome	0.0400	0.4733	36	41	35	36	0.1	0.0
GRMZM2G091560_P01 Sobic.005G143500.1.p	Aspartyl-tRNA(Asn)/glutamyl-trna(Gln) amidotransferase subunit B	Chloroplast	0.0463	0.4811	8	1	1	2	-7.0	1.0

GRMZM5G809869_P01 Sobic.003G169300.1.p	Small subunit ribosomal protein S2	Ribosome	0.0485	0.4823	16	14	22	11	-0.1	-1.0
Stress and defense										
GRMZM2G405459_P02 Sobic.004G105100.1.p	Peroxidase	Extracellular region	0.0003	0.1214	19	4	6	2	-3.8	-2.0
GRMZM2G352415_P03 Sobic.001G017100.2.p	Late embryogenesis abundant protein	Nucleus	0.0003	0.1214	19	35	17	24	0.8	0.4
GRMZM2G150893_P02 Sobic.001G277000.1.p	Peroxidase	Extracellular region	0.0008	0.1663	14	8	8	4	-0.8	-1.0
GRMZM5G806449_P03 Sobic.004G341200.1.p	Glutathione reductase, mitochondrial	Mitochondria	0.0012	0.2417	9	25	11	14	1.8	0.3
GRMZM2G026800_P01 Sobic.001G198100.1.p	PAP_fibrillin	Chloroplast	0.0012	0.2417	10	20	7	16	1.0	1.3
GRMZM5G828229_P02 Sobic.007G038600.1.p	Monodehydroascorbate reductase, chloroplastic	Mitochondria	0.0017	0.2586	29	41	5	8	0.4	0.6
GRMZM2G015285_P01 Sobic.005G172400.1.p	PAP_fibrillin	Chloroplast	0.0059	0.3075	34	37	39	49	0.1	0.3
GRMZM2G106928_P02 Sobic.007G166600.1.p	Copper/zinc superoxide dismutase (SODC)	Chloroplast	0.0079	0.3089	11	13	12	16	0.2	0.3
GRMZM5G826194_P02 Sobic.010G095200.1.p	Glutathione dehydroascorbate reductase	Cytoplasm	0.0117	0.3940	32	26	40	23	-0.2	-0.7

GRMZM2G116846_P01 Sobic.001G444500.1.p	Peroxidase (E1.11.1.7)	Extracellular region	0.0216	0.4499	11	3	8	9	-2.7	0.1
GRMZM2G120517_P03 Sobic.006G084400.1.p	L-ascorbate peroxidase s, chloroplastic/mitochondrial-related	Chloroplast/Mitochondria	0.0231	0.4504	1	8	28	34	7.0	0.2
GRMZM2G024315_P01 Sobic.003G222300.1.p	Aldo/keto reductase	Cytoplasm	0.0370	0.4658	13	20	42	46	0.5	0.1
Transcription										
GRMZM2G174757_P01 Sobic.001G291800.1.p	Translation initiation factor 3 subunit B	Cytoplasm	0.0021	0.2592	13	5	3	0	-1.6	-3.0
GRMZM2G043212_P01 Sobic.003G295800.1.p	50s ribosomal protein l13, chloroplastic	Ribosome	0.0022	0.2592	12	7	11	3	-0.7	-2.7
GRMZM2G053985_P01 Sobic.006G096600.1.p	Transcriptional repressor, ovate	Nucleus	0.0023	0.2592	0	0	8	11	0.0	0.4
Biogenesis of cellular component										
GRMZM2G007195_P02 Sobic.009G112600.1.p	Nad dependent epimerase/dehydratase	Integral component of membrane	0.0021	0.2592	13	17	9	15	0.3	0.7
GRMZM2G037177_P01 Sobic.002G395000.1.p	Dynactin subunit p25	Dynactin complex	0.0060	0.3075	8	13	7	13	0.6	0.9
GRMZM2G397247_P04 Sobic.010G255100.1.p	Nad dependent epimerase/dehydratase	Chloroplast	0.0485	0.4823	40	25	44	41	-0.6	-0.1

Transport and cell structure										
AC234515.1_FGP003 Sobic.010G224900.1.p	Tubulin	Cytoplasm	0.0109	0.3819	11	8	9	0	-0.4	-9.0
GRMZM2G420733_P01 Sobic.002G005300.1.p	Nucleoporin-related	Nucleus	0.0119	0.3969	3	0	5	3	-3.0	-0.7
GRMZM2G086636_P01 Sobic.003G155400.1.p	Gtp-binding protein sar1a-related	Intercellular	0.0195	0.4399	23	16	23	23	-0.4	0.0
Unknown										
GRMZM2G701082_P06 Sobic.010G094800.1.p	Uncharacterized protein	Nucleus	0.0005	0.1274	0	10	3	13	10.0	3.3
GRMZM2G071089_P01 Sobic.004G128600.1.p	DREPP plasma membrane polypeptide	Membrane	0.0026	0.2854	12	4	4	1	-2.0	-3.0
GRMZM2G039711_P01 Sobic.006G155100.1.p	Putative uncharacterized protein	Cytoplasm	0.0145	0.4029	16	6	12	12	-1.7	0.0
GRMZM2G093900_P01 Sobic.007G074500.1.p	4-aminobutyrate---pyruvate transaminase	Chloroplast	0.0207	0.4401	4	8	2	3	1.0	0.5
GRMZM2G058261_P01 Sobic.001G067700.1.p	Uncharacterized protein ((NAD(P)-binding Rossmann-like domain)	Chloroplast	0.0213	0.4401	4	0	7	4	-4.0	-0.8
GRMZM2G423137_P01 Sobic.004G020300.1.p	Protein of unknown function (DUF4079)	Chloroplast	0.0240	0.4559	9	12	23	28	0.3	0.2

GRMZM2G429000_P01 Sobic.006G172700.1.p	Hydrophobic seed protein	Vacuole	0.0429	0.4775	10	10	21	10	0.0	-1.1

Proteins were grouped into functional categories according to Bevan *et al*. (1998).

(-) indicates a decreased fold-change in protein abundance between water-stressed plants and control plants. Data are representative of five biological replicates.

The eight ortholgous Protein groups which are differently expressed between well-watered plants and water deprived plants are highlighted with a grey color.

The letters (WW) represents well-watered plants and (WD) represents water-deprived plants.

The top accession number represents *Z. mays* and the bottom accession number represents *S. bicolor*